High-Resolution Screening of Metabolite-like Lead Libraries

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High-resolution screening of metabolite-like lead libraries

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Section 1

Introduction

Chapter 1.1

General introduction
1.1.1 Discovery of new active pharmaceutical ingredients

1.1.1.a) Screening

The vast majority of medicines work by modulating the activity of a target protein. Therefore, the discovery of an active pharmaceutical ingredient (API) involves finding molecules which interact with that target protein. This process, called screening, is the initial step to discovering a new compound that would be achieved by administering the molecules to diseased animals (animal models), a so-called phenotypic screening [1]. The high cost, limited throughput and increasing awareness of animal welfare, catalyzed by the emergence of biotechnology, have triggered the search for alternative in vitro methods [2]. In addition, it is easier to link an in vitro assay response to a molecular mechanism of action. In in vitro assays, the target protein is present either isolated or in individual living cells. The former is often the cheapest and simplest approach [3], which, in combination with the implementation of lab automation and robotics, has led to the emergence of high-throughput screening (HTS) [2]. Conceptually, HTS is based on the screening of large numbers, sometimes up to millions, of molecules (compound libraries) with the idea that APIs are discovered by pure chance [4]. However, even in HTS, there is a strong trend to reduce the chance element by designing focused compound libraries [5]. The technological platform consists of well plates, dispenser robots and specialized spectrophotometers, called plate readers [4]. With the extensive use of HTS within pharmaceutical companies, its limitations are becoming increasingly obvious [6]. A major issue is the quality of the hits (the compounds that show interaction with the target protein) [7]. The statistical approach used results in false positives which requires all hits to be re-screened for confirmation [6]. While HTS is unmatched for large libraries of compounds, it is not as useful for efficiently screening small numbers of compounds in the confirmation screening or at later stages like in lead optimization. Additionally, the purity of the compounds screened can have a significant influence on hit quality [7]. Thus, achieving suitable screening libraries as well as maintaining them over years in different screening campaigns is challenging. Therefore, impurities and degradation products can lead to false assignment of active molecules as well. A significant effort has been put into improving, supplementing or even replacing HTS, for example with high-content screening [4], improved animal models [1] or in silico screening [6]. One of the key technologies in this context is high-resolution screening (HRS) [9,10] which can address some shortcomings of HTS in terms of flexibility and hit quality [Chapters 1.1.2 and 2.1]. Additionally, HRS is uniquely suited to screen mixtures and thus not limited to the use of pure compound libraries.

1.1.1.b) Metabolite-like lead libraries

The large compound libraries that are used in the initial screening preferably have a high chemical diversity [4], possibly within a predefined space [1]. After the hit confirmation and hit-to-lead selection [6], new libraries are created around the selected hit scaffold, the so-called lead libraries. Lead libraries are much smaller and incorporate a much narrower chemical space than the screening libraries [11]. However, both types of libraries traditionally consist of (more or less) pure compounds. An exception is presented by some combinatorial chemistry libraries [12] and natural extract libraries [13,14]. The latter originate from a wide variety of sources, including animal venoms [15] and plant extracts [16]. Due to their huge chemical complexity, these libraries mostly consist of mixtures of compounds, even if fractionation of the original sample was included.

Not only chemists can generate molecules, but also our body itself is a complicated assortment of chemical reactions known as metabolism. Amongst others, this includes dealing with potentially harmful, foreign substances in our diet, the so-called xenobiotic metabolism. This consists of different enzymatically catalyzed reactions which are intended to increase the hydrophilicity of the xenobiotics with the ultimate goal of renal or fecal excretion. The main impact thereof is in pharmacotherapy, where the phenomenon is known as drug metabolism, because APIs are modified. The metabolites of APIs are independent entities. In designing molecules, medicinal chemists often try to avoid metabolism as they fear unwanted side effects and a decreased therapeutic efficacy [17,18]. However, a metabolite may possibly show better pharmacological properties [18]. Even more so, the intrinsic tendency towards increased hydrophilicity is a real advantage. The molecules used in modern drug discovery often suffer from excessively low hydrophilicity which prevents them from entering the circulation or allows them to enter the brain [19,20]. Consequently, more polar compounds are more easily absorbed and more effectively excluded from the brain. An indirect advantage of hydrophilicity is found in the intended and unintended interactions with (target) proteins. Non-polar molecules mostly draw their affinity from van der Waals interactions which are undirected induced-dipole interactions. The unspecific nature of those interactions increases the likelihood of affinity with multiple proteins which may lead to side effects [19]. In more polar molecules, amine, hydroxyl or carboxylic acid groups form interactions like hydrogen bonds, dipole-dipole interactions or ionic interactions, requiring more specific partners in the protein. This may increase the specificity for the target, thus reducing side effects. In this context, specificity is defined as the relative affinity for the intended target versus other proteins. There are actually quite a number of examples where a metabolite ended up replacing the original active ingredient due to improved pharmacological properties [18]. Thus, the collection of metabolites derived from an active ingredient and a lead library derived from a hit are quite comparable. The scaffold is randomly (at least before establishing a pharmacophore model) modified by simple reactions yielding distinct but similar products. One aim of this thesis is, therefore, to investigate lead libraries which incorporate the positive bias of metabolism towards selectivity and hydrophilicity. Though the chemical space associated with metabolites is not easily accessed by classical synthetic approaches, there are a number of methods which are frequently employed for the generation of metabolite standards, some of which are discussed later. As our interest is not limited to actual metabolites, but extends to all products which show typical metabolic modifications, we use the term metabolite-like lead libraries. However, we use this term to describe the structural similarity of the molecules to drug metabolites and not (necessarily) to actual endogenous metabolites [21].

1.1.2 High-resolution screening

Although the term high-resolution screening (HRS) was originally introduced for on-line post-column bioassays (see Figure 1A), we use HRS to mean any of the various in vitro approaches which achieve the integration of a separation technique and a biological assay (bioassay) for screening purposes in drug discovery. The key benefit is that integrating separation and biological evaluation enables the HRS platform to handle mixtures of compounds and still be able to assess individual biological effects. Chapter 1.2 introduces important concepts and distinctions, and briefly discusses advantages and disadvantages of different HRS assay variants as well as of HRS over HTS assays. Examples are cited for the different concepts, preferably showing their use in HRS assays. A comprehensive overview of HRS methods is not attempted. The reader may refer to several recent reviews [24,25,10,9,23,26]. Although bioassays can also be set up to screen a single (or a limited number of) compound(s) for the interaction with multiple targets [27], we focus on the more common approach to develop a screening for a single target (or a small number of targets) [4]. An important distinction lies in the nature of the biological effect which is probed. This can be either biological affinity (bioaffinity) or biological activity (bioactivity). Bioaffinity assays measure the binding of an analyte to the target [Chapter 2.2]. Bioactivity assays can be further divided into biochemical assays which follow an enzymatic reaction [Chapter 2.3] and functional assays which visualize an indirect response to the analyte-target interaction [28]. Bioaffinity of an analyte is measured either by the displacement of a tracer molecule
Bioaffinity assays either rely on affinity selection of the analyte using an immobilized protein [29-31], separate free and bound tracer [32] or utilize the difference between free tracer (or analyte) and tracer-target (or analyte-target) complex with regard to mass [33], spectroscopic properties [Chapter 2.2] [34,35] or Gibbs free energy [36]. Advantages are that the inactive form of a protein can be studied and no expensive co-factors are needed (see also Chapter 2.1) [Chapter 2.2]. However, though without affinity there is no activity, binding may potentially evoke different responses in the protein. Therefore, in bioaffinity assays the direction of activity remains unknown, for example whether a binder is an agonist or an antagonist. This is the domain of biochemical and functional assays. Biochemical assays measure a biocatalytic reaction by detection of a substrate and/or the product(s) and are therefore mostly applied to the study of enzymes [Chapter 2.3]. The difference in mass [16] or spectroscopic properties [Chapter 2.3] between the substrate and the product(s) is the key to this approach. Detection of multiple reaction partners (mainly substrates and products or reactants in short) usually increases the robustness of the assay, for example by revealing the influence of unexpected side reactions [37]. While, in principle, biochemical assays can be used to screen different substrates for the same (detected) product, the more common approach is to observe the effect of inhibitors, allosteric modulators, or competing substrates on a particular reaction. Given the use of the same detection technique, biochemical assays are usually more sensitive than bioaffinity assays, because of the signal amplification by reiterative, biocatalytical reaction cycles. In proteins, which do not catalyze a reaction, for example nuclear receptors or G-protein coupled receptors, functional assays are needed to investigate the nature of the biological effect [28]. These highly complex assays are often cell-based and mostly measure an effect of some signal cascade, like calcium release [38], labeled secondary messengers [39] or protein expression [28]. Because several signal amplification steps can be involved, functional assays are usually even more sensitive than biochemical assays. An endpoint has to be chosen beforehand, meaning that effects on the target leading to different signaling endpoints are not detected. However, in many studies, the effect of the analyte on the endpoint is more interesting than the mere effect on the target itself. This is both the advantage and the drawback of functional assays, because any effect of the analyte on the endpoint detected is merely assumed to be caused by the analyte-target interaction. In this respect, better certainty is achieved, if bioaffinity is established in addition to the specific bioactivity yielded by the functional assay [28]. An almost uncountable number of assay principles is currently available. Nonetheless, a brief overview is attempted referring to categories of principles rather than individual methods. The most desirable situation for bioaffinity assays is the direct measurement of the analyte. This is especially useful for novel targets with no or little known ligands [40]. However, it requires a detection technique capable of measuring all envisioned analytes or analyte-target complexes. Though mass spectrometry (MS) [29] and surface plasmon resonance (SPR) [31] are well suited to achieve both the necessary selectivity for binders over non-binders and the broad detection, even their application often requires at least additional purification steps. For example, it is very difficult to reliably ionize the intact analyte-target complex in MS which thus often requires prior separation of the unbound analytes [29]. Therefore, a tracer is often used for bioaffinity assays. This is a binder whose bound or unbound fraction can be easily monitored. Affinity of an analyte induces competition for

**Figure 1:** Comparison of pre-column and post-column HRS setups. A) shows the specific setup used in Chapter 2.2 including an on-line post-column HRS bioaffinity assay and parallel HR-MS® detection. The setup is rather generic for on-line post-column assays and holds true for Chapter 2.3, by simply changing tracer to substrate. Variations are discussed in detail in Chapter 2.1. B) represents a unified schematic of pre-column affinity selection assays rather than a specific setup [12,22,23]. In the first step, protein and analytes are mixed so the binders form protein-analyte complexes. The proteins can be immobilized or can be free in solution during this step. Then, binders and non-binders are separated in a selection step, for example by SEC or by washing the immobilized proteins. Afterwards, the binders are released, trapped on an LC column and finally analyzed by LC–MS.
Perhaps, the most important distinction in HRS from a technical perspective is between pre- and necessary safety, synthesis and waste disposal efforts. During assay development, radioactive labeling is very costly, due to the 
14 is necessary when the assay principle does not allow to distinguish bound and unbound detected reactant is separated from other molecules before the actual detection step. This A distinction which only applies to post-column assays is whether the binder, tracer or immobilized [23]. The bound fraction of analytes is then selected and, subsequently, either where the mixture of analytes is allowed to interact with the target which can be soluble or immobilized [23]. The bound fraction of analytes is then selected and, subsequently, either directly detected, for example with SPR [31], or released from the target, for example for MS [30] or liquid chromatography–MS (LC–MS) detection [12]. Because bioaffinity is not assessed on individual compounds, but from the mixture, an excess of one high-affinity tracer or the reactant(s) is of utmost importance. The tracer only probes the binding sites and is lost and solvent evaporation is also not without risks. For example, we developed an micro-fractionated into 96-, 384- or 1536-well plates [28,41,42]. Incubation and readout of the effect signal is retained. The greatest advantage of the at-line approach is that long incubation times can be achieved with limited compromise on the chromatographic resolution in the effect signal. This advantage is accompanied by the drawbacks of an open system and a major increase in time and effort. However, in cell-based assays, the at-line HRS approach may be preferred, because of the long incubation times or because of the need to use adhesive cells. To avoid the physical stress on the cells or to enable solvent evaporation, the mixing is sometimes postponed to after the separation step (see Figure 1). Pre-column assays rely entirely on bioaffinity measurements [23], while post-column assays are also open to biochemical [Chapter 2.3] and even functional readouts [26]. In pre-column assays, the first step is an affinity separation whether the analytes are allowed to interact with the target which is soluble or immobilized [23]. The bound fraction of analytes is then selected and, subsequently, either directly detected, for example with SPR [31], or released from the target, for example for MS [30] or liquid chromatography–MS (LC–MS) detection [12]. Because bioaffinity is not assessed on individual compounds, but from the mixture, an excess of one high-affinity analyte compared to the target concentration prevents lower analyte concentrations from being detected if they compete for the same binding site. Affinity selection is not biased towards a certain binding mode or region. On the one hand, this can lead to issues with nonspecific binding. On the other hand, compounds using new (allosteric) binding sites can be detected without prior knowledge of these sites. This, the relative ease of implementation and the potentially high throughput have made pre-column bioaffinity assays commercially attractive. Consequently, the Automated Ligand Identification System (ALIS), which is based on size exclusion chromatography–reversed phase LC–MS, has become the biggest commercial success in HRS so far [12]. In post-column assays, the separation is the first step. Consequently, all following steps are applied to the pre-separated analytes meaning that they affect each analyte individually. This means that, in principle, a separate signal is obtained for every compound with (a sufficiently large) biological effect. Due to the fact that, assuming sufficient separation, every analyte enters the post-column assay separately, every HTS assay is theoretically convertible into a post-column assay. For more details on post-column assays, refer to Chapter 2.1.

1.1.3 Modification methods in metabolism studies

For the study of the metabolism of an API, different model systems are used to predict the changes of an API after ingestion by patients or healthy volunteers. Most commonly, this is attempted by the use of animal models, cultured human hepatocytes or in vitro incubations with (human) liver fractions [44,45]. However, none of these methods is particularly suitable to provide sufficient amounts of material to further investigate the chemical and biological properties of the metabolites. Therefore, an increasing number of methods is proposed to augment metabolic studies by producing metabolite standards. This is necessary, because the many reactions observed in metabolism are not easily reproduced by classic organic synthesis approaches. Oxidative metabolism yields a broad variety of reactions, depending on the structure of the active ingredient. Mainly, N- and O-dealkylation, dehalogenation, epoxidation, carbonyl oxidation and N- and S-oxidation are observed [46]. Especially the introduction of hydroxyl groups to non-activated carbon based on radioactively labeled tracers where bound and unbound fraction need to be separated because they show no difference in radioactivity [28]. Due to the highly dynamic nature of bioaffinity, washing away the unbound fraction, while retaining all bound tracer, is not trivial. Additionally, heterogeneous assay are usually much more labor intensive and error prone than their homogeneous counterparts [32]. Consequently, state-of-the-art post-column HRS assays are usually homogeneous assays. Assays can also be distinguished on whether the separation step is operated in the same flow system as the bioassay. If this is the case, we speak of an on-line or continuous-flow assay [9]. The (virtually) closed system and the retention of a peak shape for the effect signal result in many advantages. Chapter 2.1 deals extensively with on-line assays. On the other end of the spectrum stands the classical HTS assay were each separate step involves liquid handling [4]. However, next to on-line approaches, HRS also utilizes hybrids called at-line assays [9]. These are based on post-column high-resolution micro-fractionation. After the separation, the effluent is mixed with the bioassay reagents and the mixture is then micro-fractionated into 96-, 384- or 1536-well plates [28,41,42]. Incubation and readout of the well plates are conducted like in HTS. If the fractionation is conducted at sufficiently high resolution, the chromatographic peak shape of the effect signal is retained. The greatest advantage of the at-line approach is that long incubation times can be achieved with limited compromise on the chromatographic resolution in the effect signal. This advantage is accompanied by the drawbacks of an open system and a major increase in time and effort. However, in cell-based assays, the at-line HRS approach may be preferred, because of the long incubation times or because of the need to use adhesive cells. To avoid the physical stress on the cells or to enable solvent evaporation, the mixing is sometimes postponed to after the fractionation. However, in this way, the efficient mixing of the on-line system is lost and solvent evaporation is also open to risks. For example, we developed an HRS platform where parallel MS detection was coupled at-line via microfractionation to three parallel bioassays; a functional assay for the human histamine H$_2$ receptor and two radioligand binding assays which assessed selectivity of the H$_2$ ligands over histamine H$_3$ receptor binding [28,43]. MS is an integral part of state-of-the-art HRS platforms [9]. Next to exploiting the excellent flexibility of MS between general and specific detection for the effect detection in bioassays, MS is used as a means of implementing rapid structure elucidation into HRS platforms [Chapter 4.1]. It additionally allows the detection of analytes without biological effect, though this ability is limited to certain HRS setups [Chapter 2.2]. For example, the necessary selection step between bound and unbound analytes in pre-column assays prevents detection of analytes without bioaffinity [30]. Though this can be advantageous with MS based bioassay readouts, because it significantly reduces the complexity of the mixture prior to analysis, it limits the usefulness of these assays for mixtures of unknown analytes.
moieties is a great synthetic challenge. Because the methods, used for metabolite standard
synthesis, offer access to a unique chemical space and are often scalable to preparative dimensions, this thesis applies some of them to the generation of metabolite-like lead libraries (see Chapter 1.1.1.b).
As mentioned, an increasing number of these modification methods are available and this section gives some examples, mainly focused on the methods actually used in this thesis (see Figure 2). For a better overview, the methods are divided into chemical, electrochemical, biocatalytic and photochemical approaches. Though the methods are logically separated by the nature of the (initial) redox agent, with respect to other aspects such as reactions, driving forces or even the ultimate redox species, the division may be artificial.

1.1.3.a) Chemical oxidation

Organic synthesis knows many oxidizing agents and some of them have been used in metabolism studies such as hydrogen peroxide [47], potassium permanganate [48] or sodium hypochlorite [49]. In classic synthesis, the oxidation aims at an existing functional group and the power of the oxidizing agent is tightly controlled in order to direct the modification to this site. However, enzymatic catalysis, which underlies metabolism, is much more controlled by proximity to the catalytic groups, e.g., the heme group in cytochrome P450s (CYPs), than by redox potentials [50]. Therefore, for the generation of metabolite-like lead libraries, the side reactions obtained with strong and thus less selective oxidizing agents can be of great interest.

Though hydrogen peroxide by itself is not a strong oxidizing agent, it can be activated, for example thermally, photochemically or by metal catalysis [51]. The reactive species is the hydroxyl radical. Additionally, hydrogen peroxide can yield epoxidation of electrophilic alkenes under basic conditions [52]. In the context of drug metabolism studies, hydrogen peroxide has been used, amongst others, to achieve S-oxidation [47,53,54], aromatic and aliphatic hydroxylation [55], formation of aliphatic ketones [55], N-oxidation [56-58], N- and O-dealkylation [57,58] and carboxylic acid formation [58]. Furthermore, metallo-organic catalysts have been employed for the generation of metabolites [59]. These metalloporphyrins are meant to mimic the active state of the CYP coenzyme Protoporphyrin IX. They can be operated with molecular oxygen and reducing co-factors, but are often also used in combination with chemical oxidants like hydrogen peroxide or sodium hypochlorite. This opens a grey area between catalytic activation of hydrogen peroxide and catalytic, metallo-organic oxidation with hydrogen peroxide regeneration.

1.1.3.b) Electrochemistry

Electrochemical conversion (EC) has been extensively explored [60] and in recent years has found increasing use in metabolic studies. Due to the low matrix content of the reactions in EC, it has become very popular in combination with MS and LC–MS, and has even led to hyphenated EC–MS and EC–LC–MS systems. The field has been extensively reviewed by the groups of Karst [59,61-63], Bischoff/Brüns [64,65] and Jurva [66]. EC can mimic a wide range of metabolic reactions, such as aromatic and aliphatic hydroxylation, N-, P- and S-oxidation, N- and O-dealkylation, alcohol oxidation, dehydrogenation, epoxidation and dehalogenation [61,64]. Additionally, it has been used for the generation and study of reactive metabolites, such as quinones and quinonemines. Prominent examples are N-(4-hydroxyphenyl)acetamid (Paracetamol), amodiaquine and clozapine [67].

The EC reaction is driven by adding (reduction) or removing (oxidation) electrons to/from the substrate at the electrode surface. Two reaction classifications are distinguished in EC as well as with CYP reactions: single electron transfer oxidation and hydrogen atom transfer/abstraction [64]. Additionally, ozonolysis might occur at high voltages [68].

1.1.3.c) Biocatalytic approaches

Because in enzymes the catalytic group is presented in the context of a complex, three-dimensional active site in which the substrate has to fit in one (or a limited number of) specific orientation(s) [50], biosynthetic approaches offer an unmatched product selectivity, especially when it comes to chiral synthesis [69]. A variety of enzyme classes has been employed for biocatalytic purposes including aldolases, lyases, dehydrogenases, reductases, transaminases, esterases, oxidases, proteases and lipases [69-74]. Viewed as “by far the most dominant and important” enzymes in drug metabolism, the CYP superfamily has received specific attention in drug metabolism studies [46]. CYPs are capable of catalyzing most of the reactions of oxidative metabolism (see above). Very promising in bioanalysis is a CYP from Bacillus megaterium, named BM3, which shows a wide substrate spectrum, a high solubility, high expression levels in E. coli and a high catalytic efficiency [75,76]. The latter is especially important, because CYPs use the expensive co-factor nicotinamide adenine dinucleotide phosphate (reduced form: NADPH). Although its consumption can be reduced from stoichiometric to catalytic amounts by employing a co-factor regenerating
system based on glucose or glucose-6-phosphate and their corresponding dehydrogenases, this presents a significant hurdle in large-scale application [77]. The versatility of employing CYP BM3 for the biocatalytic conversion of APIs is demonstrated in a recent review [76].

1.1.3.d) Photochemistry

Photochemistry, meaning the use of light to activate chemical reactions, mainly acts through the formation of radicals by homolytic cleavage [78]. While visible (VIS) light with between 167 kJ·mol⁻¹ (red) and 293 kJ·mol⁻¹ (blue) is limited to weak bonds, ultraviolet (UV) light at 586 kJ·mol⁻¹ strongly decomposes organic molecules. For this reason, most applications use visible light. Next to radical mechanisms, light can induce electrocyclic reactions. Light- and thermally-induced electrocyclic reactions start from an exited state or the ground state, respectively. The difference in orbital geometry between the two states results in a different stereochemistry [79]. Classical, synthetic applications of photochemistry include polymerization, halogenation of alkanes and carbon-carbon bond formation using organo-tin reagents [78]. In the pharmaceutical industry, another important aspect of photochemistry is known as photostability which refers to the light-induced degradation of active ingredients during production and storage of a drug [80,81]. Similar to metabolism, degradation can have a huge influence on efficacy and side effects of a formulation. In addition, light-induced degradation can also occur in vivo during the use of cosmetics [82] or pharmaceuticals [83].

With respect to the radical mechanism, photochemistry [78] shares some common ground with hydrogen peroxide [51], electrochemical and CYP oxidation [64]. Light and EC have the advantage that the energy input is tunable by changing the wavelength or the voltage, respectively. The radical and electrocyclic reaction mechanisms in photochemistry allow rearrangements in the scaffold of the molecule which are not accessible by the other methods [84].

1.1.4 Structure elucidation in mixtures using mass spectrometry and nuclear magnetic resonance spectroscopy

For the structure elucidation of small, organic molecules a wide variety of methods is available [85,86]. They include the classical spectrophotometric techniques like UV/VIS absorption, fluorescence and phosphorescence emission spectroscopy, and vibrational approaches like infra-red and Raman spectroscopy. Additionally, scattering techniques like X-ray or neutron scattering are used. However, the focus is on mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) which dominate today's routine structure elucidation of small molecules. Special attention is paid to structure elucidation in mixtures which is one of the main challenges in this thesis.

1.1.4.a) Mass spectrometry

For MS, mainly two developments have enabled structure elucidation. Firstly, the high mass accuracy of the high-resolution (HR) mass analyzers [87], i.e., sector field, time-of-flight (TOF), Orbitrap [88,89], and Fourier-transform ion cyclotron resonance, allows the determination of the elemental composition of ions [90,91] [92]. Secondly, fragmentation reveals functional groups and residues by observing specific neutral losses and allows interpretation of their connectivity. Classically, fragmentation was achieved during electron ionization (EI) with the risk of missing the molecular ion [87]. After the introduction of the soft ionization techniques, especially electrospray ionization (ESI), fragmentation of the protonated molecule had to be achieved in the mass analyzer. This fueled the development of tandem MS and ion trap MSⁿ. Tandem MS instruments can be divided into unit-resolution instruments, like quadrupole–linear ion trap and triple-quadrupole, and (hybrid) HR instruments, like quadrupole–TOF, ion trap–TOF, linear ion trap–orbitrap, quadrupole–orbitrap, and quadrupole– linear ion trap–Fourier-transform ion cyclotron resonance instruments [92] [93]. Obviously, the combination of high mass accuracy and fragmentation makes the HR tandem MS instruments ideally suited for structure elucidation. Nowadays, a kaleidoscope of fragmentation techniques is being developed, but for small molecules collision induced dissociation (CID) is still applied almost exclusively [94]. However, uncertainty in the fundamental understanding of the ESI process [95] and the reactions governing CID-fragmentation (especially for small molecules), as opposed to the thoroughly elucidated main fragmentation reactions of the radical ions, for example the α-cleavage and the McLafferty rearrangement [85], present a serious hurdle in modern MS structure elucidation. Additionally, there is a large variation between different instruments in the actual energy deposited to the ions by CID, which varies between 1 and 100 eV for ion trap and quadrupole based fragmentation [94], while in ESI a standard setting of 70 eV is applied. Therefore, almost every ESI instrument produces different MS/MS spectra of the same analyte, especially with respect to the relative abundance of product ions. A fundamental difference is found between ion trap geometry collision cells and those with quadrupole geometry. Quadrupole geometries are limited to one collision experiment per cell while ion traps can perform iterative fragmentation cycles until the number of ions falls below the detection limit. Therefore, ion trap instruments allow a much more reliable interpretation of precursor-product ion relationships [96].

The advent of tandem MS required the development of various data acquisition strategies to fully exploit its possibilities. The most straightforward is the product-ion analysis mode. Herein, a precursor ion is selected, fragmented and a mass spectrum of the fragments collected. Two other widespread strategies have greatly contributed to the success of triple quadrupole instrumentation: Selected reaction monitoring (SRM) for specific CID-reactions by selecting one precursor ion in Q, and one fragment ion in Q₁ for each reaction. It is the most popular method for targeted quantitation, probably because of the dramatically increased speed, selectivity, and data reduction compared to a full-spectrum analysis. The neutral loss analysis mode searches for a specific m/z difference between precursor and the product ion, thus for a neutral loss, and it is often used to monitor classes of molecules which show the same specific neutral loss. Because neutral loss analysis shows all class compounds regardless of the precursor m/z, as long as the neutral loss is retained, it can also detect (some) unknown molecules. Nowadays, product-ion analysis is becoming increasingly fast and sensitive, e.g., in quadrupole–TOF instruments. CID-induced neutral losses may also be monitored by data interpretation of product-ion analysis spectra. Thus, combining the strength of HR-(tandem)MS in structure elucidation with the quantitative interpretation of CID-reactions or neutral losses is an emerging approach [97]. A powerful tandem MS data acquisition strategy for monitoring unknowns is data-dependent acquisition. First, a full spectrum without fragmentation is collected and processed in real-time. Based on pre-selected criteria, the software selects the most abundant ions to be fragmented and collects their MS/MS spectra. For ion trap fragmentation this can be an iterative process (MSⁿ). In this way, the process of identifying unknown ions and collecting fragmentation data for structure elucidation can be greatly automated. The most important breakthrough with respect to MS analysis of mixtures was certainly the hyphenation of MS to separation techniques [98,99], most importantly gas chromatography (GC) using EI and LC using ESI. This hyphenation does not only enable the detection of individual isobaric and isomeric compounds, but it also significantly reduces the occurrence of ionization suppression issues in ESI. Fortunately, modern, reversed phase (RP) LC and ESI-MS are very well compatible [99]. In addition, LC–HR-MS resolves isobaric compounds which have not been separable, but issues with isomeric ions cannot be elevated by HR-MS alone. In some cases, fragmentation can help in the discrimination of isomers. When standards are available, fragmentation patterns on the same MS instrument can be compared to that of the unknown. Recently, the hyphenation of ion mobility
specrometry and MS has received increasing attention accompanied by the introduction of new commercial instrumentation [100]. Because the drift-time is usually on the scale of the MS experiment or even shorter and separation is based on size and shape rather than on the physicochemical properties employed in GC and LC, IMS might offer an interesting secondary separation dimension. However, in contrast to separation techniques, IMS does not reduce ionization suppression as it is positioned after the ionization step.

There are a number of data interpretation methods which were specifically developed for the analysis of unknown mixtures of related compounds such as they occur, for example, in metabolomics. The profile group approach [101,102] assumes that the identity of one compound in the mixture is known and that this compound is related by simple chemical modifications to the other compounds in the mixture, for example an active ingredient and its metabolites [102] or a substrate and its multiple products from a shotgun synthesis approach [42]. Firstly, the fragmentation of the standard (parent drug) is thoroughly analyzed and the MS instrument is intended for the detection of the same compound in the fragmentation tree is established. A number of structurally informative fragment ions are selected as profile groups. Then, the fragmentation trees of all analytes are measured and compared to that of the standard, following mainly the profile groups. The difference in elemental composition between the analyte and the standard is found back in some profile groups, which is evidence that the molecule is modified in the corresponding part of the molecule. The difference depends on the modification, for example, oxygenation and dehydrogenation result in $\Delta m = +16$ Da or $-\Delta m = -2$ Da, respectively. Thus, the site of modification can be narrowed down. In addition, changes in (relative) retention time in RPLC can be used to distinguish the isomeric modifications of common metabolic reactions, like hydroxylation and N-oxidation [90].

Another sensitive technique for structure elucidation and fast enough for real-time collection of structural data in LC–MS$^n$ combinations, LC–MS or GC–MS are therefore often employed as the first line of structure elucidation/confirmation [103]. Unfortunately, modifications can rarely be pinpointed with single atom accuracy. As a result, NMR is often employed at a later stage to elucidate the exact position of the modifications [Chapter 3.2] [104]. However, NMR requires much more material than MS.

### 1.1.4.b) Nuclear magnetic resonance spectroscopy

The basis of the structure elucidation of a small, organic molecule by NMR is usually a proton spectrum (‘$^1$H-NMR’) [85,105,106]. This can already reveal a lot of information about the structure of the molecule. The chemical shift of a proton indicates the hybridization of the connected carbon atom and the proximity to a heteroatom. Integration reveals the relative number of chemically equivalent protons which, combined with the hybridization information from the chemical shift, establishes the connectivity of the associated carbon atom. Analysis of the J-coupling pattern hints to the number of neighboring protons while J-coupling strength indicates their distance and steric features, such as E/Z isomerism, and can be used to match J-coupling partners. The signals of exchangeable protons are usually broader than the other signals, if they are not eliminated completely by efficient exchange with the deuterated solvent.

For the structural analysis of more complex molecules, homonuclear $'H$ correlation (2D-$^1$H-$^1$H-NMR) spectra are often useful [85,105,106]. They can reveal more structural information, but are less sensitive or require longer measurement time. Correlation spectroscopy (COSY) reveals the interaction of J-coupling partners which is especially useful when several J-couplings of similar strength were observed in the $'H$ spectrum. As a variation of COSY, total correlation spectroscopy (TOCSY) reveals not only direct coupling, but also interactions of couplings, thus revealing so-called spin systems. Instead J-coupling interactions, nuclear Overhauser effect spectroscopy (NOESY) and rotating-frame nuclear Overhauser effect spectroscopy (ROESY) reveal spacial proximity of protons by employing dipole-dipole interactions.

Protons are not the only nuclei which can be followed by NMR. Not surprisingly, carbon, the backbone atom of organic molecules, is the second most commonly detected element [85,105,106]. However, its most abundant natural isotope $^{12}$C is not NMR active, leaving only $^{13}$C with ca. 1% natural abundance. Consequently, $^{13}$C-NMR is much less sensitive than $^{1}$H-NMR. However, chemical shifts are more widely distributed, leading to higher resolution, and quaternary carbon atoms are revealed which are obviously hidden in $^{1}$H-NMR. Heteronuclear $^{1}$H-$^{13}$C correlation NMR also suffers from the low $^{13}$C abundance and is therefore even less sensitive than 2D-$^1$H-NMR. Examples are heteronuclear single-quantum correlation (HSQC) and heteronuclear multi-bond correlation (HMBC) which reveal short-range or long-range $^{1}$H-$^{13}$C or $^{1}$H-$^{15}$N J-coupling partners, respectively.

Heteroatoms are also used in NMR of small molecules, mainly $^{15}$N, $^{19}$F and $^{31}$P [85], and sometimes employed in correlation experiments like $^{19}$F-$^1$H [107], $^{19}$F-$^{13}$C, $^{19}$F-$^{15}$N [108], and $^{1}$H-$^{1}$N [109].

LC–NMR is an important development in mixture analysis by NMR though the less straightforward hyphenation has so far prevented the same widespread use which LC–MS has seen [110]. The sensitivity issue usually also prevents real-time NMR detection of LC. LC–NMR systems often operate with a stopped-flow approach and/or with an intermediate solid-phase extraction (SPE) step, decoupling the chromatographic from the detection time scale and (in SPE) additionally allowing solvent exchange and analyte pre-concentration [111,112]. The increasing resolution of NMR instruments has also enabled the direct detection of mixtures by NMR. Today, it is possible to analyze even complex biological samples [113]. Simplification of the complex spectra by specialized pulse methods can contribute to resolving overlapping signals. The effect is similar to the transition from EI to ESI in the way that information is sacrificed in order to strengthen the data that remains. Simplification is achieved by canceling the influence of J-couplings on the spectra which identify sharp signals and thus greatly simplifies and thus greatly simplifies and thus greatly simplifies the fragmentation trees in complex samples. It is routine to decouple different nuclei (heteronuclear decoupling) [114], for example protons in $^{13}$C-NMR. However, the decoupling of the same nuclei (homonuclear decoupling), for example protons in $^{1}$H-NMR, is subject to ongoing study [115] and especially interesting for the NMR analysis of mixtures.

Miniaturization is a promising approach to enhance the sensitivity of NMR. By reducing the detection volume without equally reducing the concentration sensitivity, an increase in mass sensitivity is achieved. While sample pre-concentration, e.g., by SPE, becomes even more crucial, the total amount of sample material used can be decreased by ca. 100-fold in some cases. Different geometries have been proposed to achieve a well focus r-field [116,117].

Despite all the structural information that can be obtained from the various NMR experiments, the input of information obtained with MS experiments greatly complements NMR data [111]. The simplest example is the calculation of the elemental composition from HR-MS experiments which amongst many other advantages is a much faster way of revealing heteroatoms than any method available to NMR [118]. Especially, sulfur, chlorine and bromine are readily followed by MS while their NMR detection has to be called exotic at the least. Even if all structural information can be obtained by NMR alone, MS can provide a second independent confirmation and exclude symmetric dimeric structures. Additionally, because MS is generally faster and more sensitive than NMR, the same information can be obtained by MS earlier on and more economically in a structure elucidation procedure.

Due to the more straightforward hyphenation of LC and ESI-MS, this is especially true where separation is needed, thus for LC–MS vs. LC–NMR [110]. However, because NMR has much more possibilities to elucidate structural details and even conformational questions, LC–MS and NMR have become a dream team of structural analysis for small, organic molecules [104,111,112].
References
Chapter 1.2

Scope
Figure 3: The HRS workflow integrating modification, separation, affinity assessment and structure determination. This approach is used in different forms throughout Chapter 3. The library generation was explained in Chapter 1.1.3. Sample preparation beyond HPLC was only necessary for biocatalysis [Chapter 3.2]. Different forms of pre-screening were applied to select only potentially interesting libraries. The compromise between information content and sample throughput varied due to varying sample numbers. The heart of the strategy is the HRS system allowing simultaneous separation, affinity measurement and structural analysis [Chapter 2.2]. Also included are the example steps for further evaluation of the new leads demonstrated for the biocatalysis libraries [Chapter 3.2]. Upscaling and purification allow additional structural information from NMR and a quantitative comparison of affinities, for example by IC₅₀ values.

The aim of this thesis is to investigate the possibilities for the integration of innovative synthetic methods into analytical platforms for drug discovery (see Figure 3). A strong focus of this work is the advancement of existing and the development of novel analytical methodology which would be demanded and inspired by this challenging objective. More specifically, we studied workflows and developed platforms which integrate innovative, analytical-scale analyte modification methods with HRS technology, MS structure elucidation and (miniaturized) NMR. This will make neglected chemical space available early on in lead optimization and might contribute to a more efficient drug discovery cycle, if boldly implemented in novel screening paradigms.

Chapter 1.1 introduces important concepts starting with general aspects of the discovery of API, for example screening. At the core, it introduces several approaches to HRS and rationalizes the focus of this thesis on on-line post-column assay formats. As structure elucidation has become an integral part of state-of-the-art HRS platforms, structure elucidation by MS and NMR is introduced with a special attention to the analysis of mixtures. Building on the introduction of these concepts, Chapter 2.1 describes how to set up a state-of-the-art HRS platform and a successful HRS campaign. It gives practical tips on optimization and validation of such platforms, warns of common pitfalls, and introduces specialized concepts of data analysis.

Chapter 2 is dedicated to the development of HRS assays for the drug targets encountered in this thesis, being p38 mitogen-activated protein kinase α (p38α) and soluble epoxide hydrolase (sEH). The former is widely investigated as anti-inflammatory and analgesic target, and the latter presents an emerging target for therapy of inflammation, pain and cardiovascular disease. Chapter 2.2 describes the development of the first reported on-line post-column bioaffinity assay for an enzyme target. Decreased stability as compared to more robust protein targets presented a challenge and the generally high lipophilicity of p38α inhibitors inspired novel solutions. While for sEH a more classical approach, based on homogeneous detection of a fluorescent product, was chosen, the benefits of HRS in the analysis of metabolic mixtures catalyzed the development of this assay [Chapter 2.3]. Chapter 3 deals with the integration of the innovative synthetic methods described in Chapter 1.1.3. Chapter 3.1 reports on the direct integration of flow-through EC into the HRS setup described in Chapter 2.2. This promises superior reproducibility of the combined modification, separation, affinity assessment, and structure elucidation approach. As a significant number of samples either lacking conversion or showing redundant product profiles were expected, a suitable pre-screening preceded all HRS analysis in Chapter 3.

Implementation of LC–MS for pre-screening (Chapter 3.1) provides more information, but is more resource intensive than (UP)LC–UV (Chapter 3.2 and 3.3). The compound modification with BM3, described in Chapter 3.2, offered the largest potential sample variation due to the number of tested BM3 mutants. Therefore, UPLC–UV pre-screening was applied. After selection of some favorable biocatalysts by HRS, BM3 catalyzed production was scaled up and the main products further probed for structural details and IC₅₀-values by NMR and HRS, respectively. Chapter 3.3 expands the combined modification, separation, affinity assessment, and structure elucidation approach to the application of chemical (hydrogen peroxide) and photochemical (visible light) conversion. It also compares the different techniques, identifies strengths and weaknesses, and discusses the usefulness of
the approach in lead optimization and generation of metabolite standards. Chapter 4 is dedicated to structure analysis in HRS. While Chapter 4.1 deals with MS as an integral part of HRS platforms, Chapter 4.2 explores an interesting solution to the sensitivity gap between HRS and the desirable structural refinement by NMR. Chapter 4.1 describes in detail the structure elucidation of the kinase inhibitor drug-like lead libraries by MS. On the basis of examples, general aspects related to MS structure elucidation in HRS are discussed as well as interesting observations of fragmentation pathways and gas-phase ion and radical chemistry. Chapter 4.2 shows the evaluation of EC hyphenated via SPE to stripline-NMR, the latter being a novel, miniaturized NMR approach with nanoliter sample volume detection. Next to compatibility with analytical scale samples, the integration achieves advantages in the detection of reactive products, as demonstrated in Chapter 3.1. Finally, Chapter 5 describes the quantitation and structure elucidation of APIs and their conversion products by high-temperature liquid chromatography (HTLC) – inductively coupled plasma (ICP) MS (HTLC–ICP-MS) and HTLC–ESI-MS, respectively. Although, ICP-MS has not been employed in HRS so far, the technique is an interesting candidate for integration into novel HRS platforms. The integration of analyte quantitation into HRS, for example by ICP-MS, would complete the information package desired in many types of studies [1]. The low and constant organic modifier content of HTLC is beneficial for both ICP-MS [Chapter 5] and HRS assays [2], promising an interesting synergy. We report the sensitive quantitation of bromine or chlorine containing conversion products produced by HLM, BM3 and EC from two kinase inhibitors, including a complete mass balance for EC derived samples. Furthermore, ESI-MS\textsuperscript{n} structure elucidation was achieved and correlated to the quantitation results.

References
In this chapter, we want to shed light on the different aspects that are involved in a successful high-resolution screening (HRS) campaign. We limit the discussion to homogeneous on-line post-column assays, but many of the aspects are interesting to users of all-line assays as well. As discussed in Chapter 1.1.2, heterogeneous assays are very laborious and should thus be avoided. We use certain terms like bioassay or biochemical assay in the way defined in Chapter 1.1.2 despite their much broader meaning. The most obvious requirement for HRS is a suitable, robust and sufficiently sensitive analytical platform. Therefore, the setup of such a platform is the first topic [Chapter 2.1.1]. It includes practical tips to design a state-of-the-art analytical system for HRS. The second part [Chapter 2.1.2] is dedicated to data analysis which needs special attention due to the innate complexity of the data obtained in HRS. Furthermore, certain concepts in HRS data analysis, some of which are still under development, are just a bit different than in high-throughput screening (HTS) or in conventional chromatographic detection. These concepts are needed to fully harness the advantages of HRS platforms over HTS platforms, but awareness of sources of error is critical, especially where the concepts are not fully developed yet [Chapter 2.1.2]. Screening campaigns at the scale realized in HTS have rarely been realized for HRS (except [1]), in fact to the best of our knowledge none including post-column HRS assays. Therefore, many lessons remain to be learned.

2.1.1. The high-resolution screening platform

An HRS platform consists of four essential parts, as exemplified in Figure 1. At the core is a separation setup which allows the different analytes of a mixture to be detected individually by separating them in time. In order to allow two parallel detection techniques, one for structure and one for effect analysis (affinity, activity or functional), the effluent of the separation has to be divided. This is achieved by a flow split in the second part. The third and fourth part constitutes the two parallel detection techniques being the bioassay detection and mass spectrometry (MS), respectively. The bioassay detection reports the effect, by which we mean the bioactivity or bioaffinity towards the target protein. MS gives information about the chemical composition and structure of the analytes. These four stages are scrutinized subsequently.

2.1.1.a) Separation

We identified the purpose of the separation step as being the separation of the different analytes in a mixture on the time scale. In other words, we seek to obtain a virtual chromatogram, virtual because we do not (necessarily) subject the eluent to a detection method yet. An efficient separation is desired meaning as many fully separated compounds in as little time as possible. Thus, standard state-of-the-art chromatographic or electrophoretic equipment should be utilized. While this point is of course true for any separation, there are significant differences to separations with more classical detectors like ultra-violet/visible spectrophotometers (UV) which can mainly be attributed to the very specialized bioassay detection. We focus on high-performance liquid chromatography (LC) as other separation techniques, e.g., incorporating electrophoretic separations, are still rare [3]. The most important stepping stone is the innate incompatibility of many classical LC mobile phase constituents (and flow rates) with the bioassay detection. The main reason for this incompatibility is the unfolding/denaturation of the target protein which is triggered by excessive concentrations of organic modifiers after post-column mixing of effluent and protein [4]. Additionally, extreme acid, salt or base content or immiscibility of non-polar solvents with the aqueous buffers used in the bioassay detection could interfere when using for example ion-exchange [5] or normal-phase LC, respectively. Fortunately, standard RP-LC solvents like acetonitrile and especially methanol usually only produce unfolding at higher concentrations [6]. Thus, compatibility can often be achieved by suitable dilution of the eluent with the bioassay reagent solutions. If several reagent addition steps are involved, the compatibility has to be achieved in the step in which the target protein is added, usually the first, as once unfolded protein molecules often remain denatured even after additional dilution. For example, the sEH bioassay features a ca. 1:11 dilution in the first enzyme mixing step, but only a 1:0.15 dilution when the substrate is mixed in [Chapter 2.3]. In the p38α bioassay, the influence of the gradient on the baseline is much more pronounced, because the first mixing step is 1:5 though the total mixing dilution (Dm) is with 1:9 similar that in the sEH assay (Attention: The baselines are straightened by background subtraction.) [Chapter 2.2].

However, dilution is often an important challenge in HRS. Dilution reduces the final concentration at the point of detection and thereby reduces the sensitivity of the HRS platform. There are two dilution steps involved for the bioassay detection. The first one is chromatographic dilution ($D_c$). It is a result of diffusion and convection which transforms the initially homogeneous injection plug into a concentration gradient by mixing with the solvent flow. The result is the (nearly) Gaussian signal distribution so typical for chromatographic separations. Depending on the interplay between injection volume ($V$), flow rate in the bioassay detector ($u_{bio}$) and void volume, the maximum concentration of the analyte is diluted and the signal height is reduced. Though we can consider increasing the ratio of $V$ to $u_{bio}$, this has a negative influence on the separation due to the consequential increase in peak width. The second dilution $D_m$ was already mentioned above. It is exactly the same dilution that appears when sample and reagents are mixed in a beaker or well plate, for example in HTS. $D_m$ exclusively occurs in the bioassay detection. Firstly, electrospray ionization (ESI) MS does not usually need reagents or suffers as much from incompatibility with LC. Secondly, even if a makeup flow is desired, the reduced ionization efficiency at higher flow rates can be more than compensated by suitable additives and/or an increased organic modifier concentration. We have a more quantitative look at dilution in the respective data analysis section (Chapter 2.1.2.a)). Suffice it to say for now that total dilution is usually in the order of 50 to 100 times, therefore thus far impairing the HRS measurements of low concentration samples, for example from clinical or animal studies.

We have discussed compatibility of the separation with the biochemical detection, but compatibility with MS is equally important, though usually easier to achieve. Surely, it is superfluous to recap here the hyphenation of HPLC and MS as there are excellent books and reviews on that topic [7,8]. However, one has to be aware of the many mobile phase
additives that are used to enhance ionization efficiency. For example, formic acid (FA) is often used in concentrations of 0.1% to 1% (v/v) to enhance ESI+ efficiency. FA, like any other acid, salt or base, can result in two major interferences with the bioassay. Firstly, the pH of the bioassay which is crucial for the protein-ligand interaction can be affected. Secondly, interaction of any kind, for example ionic or hydrophobic, with the protein can denature its structure. This adds to the influence of the organic modifier and might lead to more unfolding. In Chapter 2.2, for example, even 0.1% (v/v) FA in the mobile phase in combination with methanol caused unacceptable protein unfolding. In this case, reducing the mobile phase content of FA to 0.01% (v/v) resulted in a sufficient compromise between bioassay compatibility and enhancement of ESI+ efficiency.

The influences on the bioassay described above are less critical with isocratic elution LC, because they are at least constant. When gradient chromatography is used in HRS, this leads to changing assay conditions during the chromatographic run, due to the changing modifier content. Mainly, the assay window (the maximum signal) is reduced with increasing protein unfolding. Furthermore, in assays based on signal reduction, the high signal baseline decreases as a consequence of protein unfolding which complicates peak detection and integration. On the one hand, this can be prevented by increasing $D_u$ to the point where the change in influence is negligible compared to the noise and in return accepting the accompanying decrease in sensitivity. On the other hand, the changing assay window, as long as it sufficiently large over the whole gradient, does not prevent effect detection nor does it significantly change measured IC$_50$ values. Additionally, the increased modifier concentration reduces tailing and precipitation of excessively hydrophobic compounds in the bioassay. The tradeoff here is the increased protein concentration which ensures a sufficient assay window at higher modifier concentrations. Issues with (manual and automatic) peak integration can be minimized by subtracting a blank gradient run of every bioassay chromatogram [Chapter 2.2]. Another possible interference of the gradient, which has to be taken into account during assay development, is a change in detection properties of the analyte or tracer (in the bioassay detection), such as fluorescence wavelength, temperature, or ionization efficiency.

Two approaches have been offered to combine gradient chromatography with bioassays aiming to eliminate the drawbacks, i.e., a counter gradient system and high-temperature LC. The counter gradient system is an intricate setup which splits off half of the LC flow pre-column to a storage unit and releases it post-column in reverse order during the next run [6]. Alternatively, a second gradient LC system can be coupled post-column with which the LC gradient is pumped [9]. As a result of this arrangement the bioassay is always approximately a 50:50 mix of both LC solvents. The baseline shift during gradient elution is reduced with only a 2-fold dilution. However, the system adds some complexity to the HRS platform. Additionally, it is limited to symmetrical gradients and switching between two gradient programs requires at least once the gradient run time. Alternatively, high-temperature liquid chromatography (HTLC) employs isocratic solvent elution in combination with a temperature gradient. As solvents change their polarity with temperature, for example water reaches the polarity of methanol at around 200°C, HTLC can very effectively replace organic modifier gradients in RP-LC. Because the effluent is cooled post column, there is no influence of the gradient on the bioassay. Though temperature stability of the analytes can be an issue, HTLC offers great advantages in combination with bioassays [10]. In some cases, the solvent can be simplified down to pure water without compromising the separation allowing minimization of $D_u$ [11]. However, adsorption of hydrophobic analytes in the bioassay and MS sensitivity can become issues at these very low modifier concentrations.

In conclusion, the composition of the mobile phase is a compromise between separation efficiency, ionization efficiency and compatibility with the bioassay. The choice of the stationary phase is thus often limited by these restrictions. The flow rate and column dimensions are discussed in the following paragraph as this is, via $D_u$, closely linked to the concept of flow splitting.

### 2.1.1.b) Flow splitting

Flow splitting has two main functions: 1) to allow the parallel use of several detectors, and 2) to control the flow rate at which the eluent is provided to either detector. We first discuss principles and applications and then the attributes of a good flow split. Later, we go into detail on how to optimize the split ratio.

Flow splitting is achieved by providing more than one exit for the eluent after the separation step. The total flow rate applied during the separation ($u_{tot}$) then divides itself according to the resistance encountered. That means that the flow rate towards each exit (e.g. $u_{bio}$ and $u_{M}$) is such that the resistance or, in LC terms, the backpressure experienced as a consequence of this flow rate is the same in all lines. One way to make a simple split is to take a connector with 1+N connections where one is connected to the LC column and N is the number of desired exits. The exits are then fitted with tubing of various dimensions. When calculating these dimensions, the first decision to be made is the operating pressure of the split. On the one hand, it should be high enough to compensate for post-split pressure differences and variations. For example, at an operating pressure of 50 bar, a difference of 1 bar in two lines already causes 2% change in split ratio while at an operating pressure of 5 bar the same interference renders the split effectively useless because of a 20% error. On the other hand, the pressure from the split adds to the demand on the LC system and should therefore not be excessive. Especially with analytical and microbore LC columns the optimal flow rate for separation is often only reached at backpressures over 100 bar, standard LC equipment has an upper limit between 200 and 400 bar. Pressures of 40 to 60 bar in the flow split have proven to be a sensible compromise. Of course, the pressure is not only dependent on split dimensions but also on solvent viscosity and total flow rate. Solvent viscosity is especially tricky to handle as it changes during the chromatographic run. However, this does not influence the split ratio. For ease and safety of calculating and testing a split, it is useful to employ water as solvent. However, it is always necessary to check that the pressure is still sufficiently high for a stable split ratio at the lowest viscosity encountered in the gradient and that at the highest viscosity the combined backpressure of column and split does not exceed the system limits. The flow rate provided by the separated fraction is limited by the sum of the flow rates of the latter two. The majority of the flow is usually directed towards this detector. Though it would be possible to add a waste line to the split in order to divert excess flow, the split is the part of the HRS system most liable to failure, the possibility of which increases with the number of exits. Additionally, splits with a larger number of exits are also increasingly complicated to prepare. Therefore, the user often prefers a solution where the source to achieve compatibility without additional splitting. The influence of the split ratio on the decision for $u_{bio}$ is limited. The bioassay is usually carried out with a concentration-dependent detector, for example a fluorescence detector. However, the split ratio has no direct influence on the concentration of the analyte in the fraction of eluent that enters the bioassay. The fraction of total injected analyte mass entering the bioassay, which is equivalent to the split ratio, is of interest merely in samples considered mass limited with respect to the high absolute sensitivity of both HRS detectors [12].

There are also commercial solutions available for flow splitting, which operate by the same principles. In some variants, the split ratio is adjustable within a limited range. However, when using a costly commercial flow splitter, the integration of a sufficiently fine particle filter between column and split is advised as clogging of the split is a frequent problem especially in matrix intensive samples.

#### 2.1.1.c) Bioassay

The flow rate directed towards the bioassay ($u_{bio}$) is a result of the desired $D_u$ and the flow rates at which the bioassay reagents are mixed in $(u_{bio}+u_{M})$ (see Equation 1). This is exemplified with values taken from the sEH HRS platform in Equation 1 [Chapter 2.3]. $u_{bio}$ is preferred to be as low as possible to minimize reagent consumption, while the ratio of $u_{M}$ to $u_{bio}$ should be large enough to prevent protein unfolding (see Chapter 2.1.1.a). Both
are important as the purified protein often makes up for the bulk of the running cost of the HRS platform. There are two factors which limit minimization of the reagent flow. The flow rate needs to be exceptionally stable which becomes an increasing technical challenge the lower the flow rate is... Thus, miniaturization of the bioassay by employing micro- or even nano-fluidic instrumentation promises significant cost reduction [13].

\[
\eta = \frac{q_{\text{bio}}}{D_{\text{M}}} = \frac{q_{\text{R1}} + q_{\text{R2}}}{V_{\text{M}}} = \frac{15 \times 10^{-6} \text{ml} \cdot \text{min}^{-1}}{30 \times 10^{-6} \text{ml} \cdot \text{min}^{-1}} = 15 \frac{\mu \text{l}}{\text{min}} \quad \text{Equation 1}
\]

One strategy frequently employed to stabilize the reagent flow is the use of pulse dampeners. A main contribution to flow instability is the sinuous fluctuation resulting from the work cycle of the pump pistons. This is true provided the appropriate measures (solvent degassing and regular seal maintenance) have been taken to prevent the formation of air bubbles in the solvent delivery system. The pulses are smoothed by passing the solvent over an oil-supported, impermeable membrane in the pulse damper. For its function, it is necessary to apply high pressure to the solvent which is achieved by a restriction capillary providing 100+ bar backpressure.

We can gain distinct advantages by delivering the reagents indirectly instead of pumping them through an LC pump. This can be achieved by running the pump on deionized water which is passed through a pulse damper and restriction capillary to generate a stable solvent flow which in turn operates a superloop. A superloop is a plastic covered glass cylinder. Both ends are sealed watertight but provide waterflow through a standard LC connection. The cylinder also hosts a moveable, water-tight piston. On the inlet side, the water enters the superloop and pushes the piston. On the outlet side, the reagents are stored. By the movement of the piston, the reagents are pushed out at the same flow rate as the water enters. The reagents are then mixed with the LC eluent. By using this setup, we save reagent solution as we feed only the superloop tubing from superloop to LC connection and not the significant void volume of an LC pump with reagent. By keeping the void volume of the transfer tubing low, the equilibration time, needed to achieve a stable assay baseline, is also shortened. In addition to saving expensive reagents, the setup reduces maintenance and increases lifetime of the pump as contact with chemical and/or biological reagents is avoided. The superloop is also easily cooled by placing it in an ice bucket. This allows to run an HRS system for hours, depending on the stability of protein or reagents, without interruption.

There are a number of measures we should consider to achieve an optimal performance of the superloop. The piston should move as freely as possible within the superloop. Thus, regular greasing is essential. While filling the superloop with reagent solution and water, air bubbles should be avoided. These increase fluctuations in the outlet flow as they compress and decompress every time the piston movement is slightly hindered. Because prevention of air bubbles is not always possible, the superloop should be stored with the inlet slightly elevated. This at least prevents artifacts from release of the bubbles into the reactors during the analysis. The specific described superloop type has a pressure limit of around 10 bar. Mixing of the eluent with the reagents is achieved with a T-piece. The mixing in an online post-column bioassay is highly efficient, especially when coiled reactors are used which is discussed later. This is one of the major advantages over well plate or test tube based assays and the reason why shorter incubation times can be achieved at similar reagent concentrations [Chapter 2.3].

However, being essentially only a simple piece of PEEK, PTFE or fused silica tubing, mixing in the solution has to be devoted to the reactors in post-column bioassays. One reason is the fact that we purposefully introduce additional void volume between separation and detection. This necessarily leads to reduced separation efficiency by peak broadening. The efforts described in the following are only justified if other prominent sources of void volume are also meticulously managed. Minimal void volume connectors and T-pieces should be utilized and special attention has to be directed to avoiding ill-fitted connections. In addition, excessively large spectrophotometric detection flow cells compared to \( u_{\text{opt}} \) are a main cause of peak broadening [14]. From post-column derivatization, the coiled optical tubing reactor (coiled reactor) was adopted to reduce peak broadening. It is simply a specially ‘knitted’ PTFE tubing which induces secondary flow patterns thus increasing radial mass transfer and reducing peak broadening [15]. By increasing the radial mass transfer, the coiled reactor also contributes to the mixing of eluent and reagents. Other contributors to the deterioration of the separation are interaction between the reactor material and the analytes. In PEEK and PTFE reactors, these are mainly hydrophobic interactions. We therefore recently proposed to use chemically modified fused silica tubing for highly lipophilic analytes [Chapter 2.2]. Chemical modification is necessary, as the use of untreated fused silica results in strong dipole-dipole or ionic interactions of the free silanol groups with nitrogen containing moieties often found in drug-like molecules. Instead, by chemical modification of the fused silica, its surface properties can be tuned to minimize interactions with the respective analyte. For example, polyethylene glycol (PEG) modified fused silica tubing has been successfully used for highly lipophilic drug-like molecules [Chapter 2.2].

A disadvantage of the fused silica tubing is that its low flexibility does not allow knitting it in the same way as the PTFE tubing. Therefore, depending on whether void volume or adsorption is the main cause of peak broadening, a choice must be made between coiled PTFE or straight fused-silica reactors until such time when fused-silica reactors with the knitted-coil geometry become available. A third option, which however has been applied only in miniaturized assay formats so far, is the use of glass microfluidic chips [13, 16]. Like fused silica tubing, they can be deactivated by chemical modification [13]. On top of that, microfluidic chips consist of standard chips containing the fluidics as a lot of low pressure connections. The introduction of hair-pin curves prevents a laminar flow profile [17], and thus enables efficient mixing and avoids excessive peak broadening (see above).

For optimal performance, the inner diameter (I.D.) of the tubing should match the flow rate applied. Additionally, this results in a large surface-to-volume ratio which is favorable for the bioassay [Chapter 2.3]. Furthermore, incubation, especially for longer times at elevated temperatures, in HTS results in evaporation, which is a major source of errors in HTS. Due to the (virtually) closed system, this is no issue in HRS. Of course, minimizing the I.D. is limited by the pressure limit in an I.D. tubing. For example, the length increases with the square of the diameter reduction. An I.D. around 250 µm presents the most common compromise, but longer incubation times might require 500 µm or 750 µm I.D. tubing.

We have now discussed the delivery and mixing of a reagent with the column effluent. However, usually, there are at least two different reagents needed. On one hand, the enzyme or receptor whose interaction with or manipulation by the analytes is studied, and on the other hand, the substrate or tracer indicating inhibition of or affinity with the target protein. These two reagents might be delivered in one [13] or two separate steps [Chapter 2.2 and 2.3]. Delivering protein and tracer with the same superloop reduces the complexity and cost of the system. Whether the reagents can be pre-mixed, depends on the bioassay principle. For example, it is obviously not possible for bioactivity assays. Due to the short incubation times typical in HRS (usually ≤5 min), affinity assays can be negatively influenced as well. While binding to the protein is usually quite fast, competition reaches equilibrium significantly slower [18]. Therefore, it is often favorable for the sensitivity of the affinity/activity detection to make a first mixing and incubation step of the protein with the eluent, thus allowing fast association with the analytes, and then to introduce the substrate or tracer. Nonetheless, for binders with slow association kinetics (small kon), which do not reach equilibrium in the first incubation step, concentration sensitivity of the affinity measurement is lowered and IC50 values are underestimated. This issue may also be observed...
in bioactivity assays.

2.1.2 Data analysis

2.1.2.a) Differences between HRS and HTS

Influence of shorter incubation times

As the incubation time is generally limited to max. ~5 min by peak broadening, incubation times are often much shorter in on-line bioassays than in HTS assays. Note that this is not applicable to HRS assays based on high-resolution fractionation, as in such cases the bioassay is essentially decoupled from the LC, and thus does not have to take place within the LC timescale. One of the consequences is that competition between the analytes and the tracer/substrate in on-line bioassays is more likely to be under kinetic rather than under thermodynamic control (compare Equation 2). This can have an influence on the detected IC\textsubscript{50} values and the sensitivity of the assay. In the two superloop setup, this creates a bias towards binders/inhibitors with a slow dissociation kinetic (k\textsubscript{on}). However, as current opinion sees slow k\textsubscript{on} as favorable due to a link with better PK/PD properties, this bias seems acceptable [21]. When looking to association kinetics (k\textsubscript{on}), most binders/inhibitors reach a significant amount of binding in 30 s of pre-incubation. The sensitivity of the assay is therefore only negatively influenced in binders/inhibitors with a very slow k\textsubscript{on}.

\[ K_D = \frac{k_{off}}{k_{on}} \]

Equation 2

Slow assays might not be possible, but on-line assays usually allow quite a time reduction from HTS.

The assay signal

One difference in data analysis between HRS and HTS is the calculation of the signal which is directly linked to the origin of the reference. In HTS, the reference is represented by the control sample which gives the assay readout without any observable effect. Replicates produce a mean and standard deviation (SD) of the control, \( \mu_C \) and \( \sigma_C \), respectively. This is essentially an external reference. In another experiment, mean and standard deviation of the sample are obtained, \( \mu_S \) and \( \sigma_S \) respectively. The signal is then calculated by subtracting the means \( \mu_C - \mu_S \). In HRS, no control sample needs to be measured. The baseline of the on-line bioassay readout provides an easily accessible and highly relevant internal reference for each individual sample. Thus, the signal in HRS is identified as the peak height (\( \mu_L \)). Therefore, contrary to HTS, changes in the background do not influence the signal which results in a lower SD of the peak height (\( \sigma_L \)) compared to \( \sigma_C \). However, changes in the (theoretical) signal at full effect, the assay window, are equally uncorrected. The variation of the reference, also called the noise, is usually calculated differently in HRS than in HTS (see \( \sigma_L \)). Instead of calculating the standard deviation of the baseline, the maximum deviation of the baseline signal is calculated which is more easily accessible. This is halved to account for positive and negative deviation from the baseline. Though the noise is slightly overestimated in this way, this method is more robust in dealing with baseline changes due to gradient elution. In bioassays, there is always a (spectrometric) signal which indirectly visualizes the underlying bioactivity/bioaffinity. For correct measurement of this signal, the chromatographic detector has to be within its linear range. However, the resulting concentration-signal dependence is not linear due to the underlying biochemical interaction. If the detector response is linear, the typical sigmoidal dose-response curves result when plotting \( \mu_L \) against the logarithm of the concentration. However, the influence is not limited to the peak height. As in any chromatographic peak, the analyte molecules are distributed according to a Gaussian distribution because of the longitudinal diffusion of the initially homogenous injection plug (\( V_f \)), each data point has its own analyte concentration. This results in a distortion of
the ideal Gaussian shape in the bioassay detection. Fortunately, the sigmoidal curve has a significant pseudo-linear part, usually between 10 and 90% of the assay window, thus allowing the calculation of important peak parameters like the full width at half maximum (FWHM) (see Figure 4). However, if \( \mu_H \) is outside this pseudo-linear range, the consequences are much the same as if a classical signal would be outside the linear range. For example, if \( \mu_C \) is above 90% of the assay window, the FWHM increases as the flanks of the peak are still growing linear while the peak is not, the so-called overloading effect.

While the use of signal intensity (height) is natural to a pharmacologist, as HTS and other batch assays simply do not show peaks, separation scientists prefer working with peak areas, as these are less sensitive to fluctuations in the separation than the peak heights are. The most prominent assumption is the detection of an underlying (pseudo-) linear phenomenon. However, in order to obtain reliable IC\(_{50}\) values, we would like to acquire sufficient data points in the non-linear parts of the dose response curve (this increases the quality of the data fitting), automatically violating that assumption. As a consequence, employing the peak area of the bioassay response would create a relatively large, systematic \( y \)-error in the asymptotic region of the dose-response curve (see Figure 4). Due to the fact that the curve is asymptotic towards a \( y \)-value, the \( x \)-value error caused by distortion of the FWHM calculation is far less influential. More importantly, while the \( y \)-value error in the peak area increases continuously with increasing injected concentration, the \( x \)-value error in the calculated concentration becomes increasingly meaningless with increasing \( x \)-values. In addition, the FWHM of the pseudo-linear range might be used for the non-linear range thus reducing the \( x \)-error for the most affected data points close to the pseudo-linear range. The determination of IC\(_{50}\) values from the dose-response curve is discussed in more detail in the next section.

**Dilution calculations**

As explained earlier, there are two types of dilution, \( D_d \) and \( D_c \), whose product forms the total dilution \( D \). While \( D_d \) represents essentially the same step as the mixing of the analyte solution with the assay reagents necessary in HTS, \( D_c \) is unique to HRS. \( D_c \) is simply calculated by dividing the total flow rate through the bioassay detector (\( u_C \)) by the flow rate with which the analyte containing effluent enters the bioassay (\( u_E \)). \( D_c \) results from the transformation of the injection volume (\( V \)) into the near-Gaussian distribution (see Chapter 2.1.1.a)) and can therefore be calculated by Equation 3. With \( D_d \) and \( D_c \), we can calculate the final concentration (\( c_f \)) at \( \mu_H \) from the injected concentration (\( c_i \)), using Equation 5. It has to be remembered, however, that Equation 5 is derived for an ideal Gaussian distribution which means that the results grow less accurate the more the shape of the bioassay peak deviates from the ideal (see discussion in the previous section). This concept should be further developed in the future to include deviations from the ideal distribution, for example, by including the peak tailing factor in a revised equation.

\[
D_c = \frac{\text{FWHM}}{2} \times \frac{\sqrt{\pi \ln 2}}{V_i} \frac{u_{B10}}{u_E}
\]

**Equation 3**

\[
D_M = \frac{u_{B10}}{u_E}
\]

**Equation 4**

\[
c_f = \frac{c_i}{D_M \times D_C}
\]

**Equation 5**

**MS structure elucidation**

Most often HRS is used for the analysis of mixtures of related molecules. These are mixtures of molecules that (often) have the same or similar core structures. They can be derived, for example, from chemical or biochemical diversification approaches (Chapter 3) or they could be different molecules from one class or type of natural products [12,22]. The structure of individual compounds is identified by a combination of accurate-mass measurements and CID-fragmentation experiments (see also Chapter 1.1.4.a)). As HRS often deals with unknown compounds, data-dependent acquisition (involving switching between MS and MS\(^n\) mode) is of key importance. MS instrument settings should be optimized using an available model compound from within the compound class targeted at expected concentrations. If data-dependent precursor selection fails, the afflicted experiments have to be repeated with manual precursor selection after data processing of the MS\(^n\) data. Peptides and small proteins might be elucidated (partly) by database comparison [12] or by top-down proteomics approaches. For small molecules, the first step is deducing the elemental composition of the parent ion. To this end, a range of elemental compositions pre-selected from prior knowledge of the analyte class, modification pathways, etc. is matched to the accurate mass. Based on the stringency in matching accurate and exact mass and the range of elemental compositions allowed, one or more elemental compositions are yielded. For the fragmentation analysis, the elemental compositions can be limited to that of the precursor ion. Specific neutral losses can then be used to identify functional groups and elucidate their connectivity [Chapter 1.1.4.a)]. If several possibilities for the precursor ion exist, some wrong elemental compositions might be uncovered through proof by contradiction that is if no sensibly neutral losses connect the precursor ion with the product ions.

**Figure 4: Theoretical dose response curve.** The dots represent the curve which would be expected when using peak height as readout. The triangles at high concentrations present the systematic \( y \)-value error which would occur when using peak area instead of peak height.
Structure-affinity matching

Matching the structure and the affinity of a molecule by matching its MS and its bioaffinity response starts by aligning the extracted ion currents (EIC) of the MS detection with the bioaffinity chromatogram (e.g., from the fluorescence detector). As both chromatograms result from the same chromatographic run, one chromatogram is simply time-adjusted for the stable retention time delay ($\Delta t$) which is a consequence of differences in post-column void volume. Variations in flow rate could cause $\Delta t$ to vary. Therefore, the SD of the total retention time could be used as a boundary [Chapter 3.1]. All MS peaks with retention times ($t_{r,MS}$) within this SD around the bioaffinity peak with $t_{r,MS}$ are accepted as peaks potentially having bioaffinity. This is evidently over cautious, especially considering all measures taken to ensure flow stability in the bioassay, because the analytes usually spend significantly more time in the separation part than post-column. Additionally, variations in temperature and solvent composition influence the chromatographic retention time and not the additional SD of the post-column. However, other factors are usually more crucial than correct accurate peak matching (see below). In most HRS setups, the FWHM of the bioaffinity peaks is larger than the SD of $t_r$ let alone the deviation of $\Delta t$. Especially when dealing with overloading (see above) in either of the responses, peak maxima and consequently $t_r$-values can be difficult to identify. Therefore, we often mark both MS and bioaffinity peaks at their onset rather than at their top, because this is better defined. Nonetheless, this may result in different marks for the same compound depending on its concentration and thus in unstable retention time differences for different compounds, because the onset is further away from tr in a broad peak than it is in a sharp peak. Consequently, the variation in peak width ($\Delta t$) is another criterion for the accuracy in the peak matching and overloaded peaks have to be even more carefully assigned. If peaks are assigned on the onset, it is more or the bioaffinity response can influence this matching as well. However, co-elution of other affinity compounds and overloading of either the MS or the bioaffinity response can influence this matching as well.

2.1.2.b) Validation

Validation is the final stage of every assay development, not excluding HRS systems. In HRS, we usually start by validation of the bioassay and add the MS later for further validation of the whole setup. A good first validation step is the measurement of a number of specific assay parameters which give a quick first quality assessment of the assay and if necessary show potential for improvement. Simple measurements reveal all contributions to the background signal as well as the assay window [Chapter 2.3]. An assay window (for calculation see below) with a signal-to-noise ratio (S/N) between 50 and 100 is an indication of a good-quality bioassay, because it allows detection (S/N>3) of the flat part of the sigmoidal curve between 5 and 10% signal and quantitation (S/N>9) of (most of) the pseudo-linear part of the sigmoidal curve between 10 and 90% signal [Chapter 2.2 and 2.3]. The former greatly enhances sensitivity of the bioassay, the latter is essential to obtain good-quality IC$_{50}$ values. If sufficient S/N is not yet reached, the individual background contributions provide a clue to which part still has to be optimized. For example, if by far the largest contribution comes from the tracer, decreasing the tracer concentration or increasing the enzyme concentration are obvious starting points. It should be mentioned here that sometimes lower S/N for the assay window are preferred due to cost considerations or other limitations, excepting the loss in assay quality [13,23]. We quickly explain how two important benchmarks of bioassay quality can be calculated in an HRS setup: the assay window and the $Z'$ factor [24]. For both of them, we need $\mu_c$ at full inhibition or binding ($\mu_{c,max}$) as well as the baseline noise. For activity assays, $\mu_{c,max}$ can often be found by comparing the signal at the incubation temperature and at 0°C (where virtually no enzymatic reaction is observed). Injection of an analyte concentration which results in complete inhibition or binding is a way to find $\mu_{c,max}$ which works in both activity and affinity assays. To calculate the S/N of the assay window, we simply divide $\mu_{c,max}$ by the noise. The $Z'$-factor as defined for HTS is given in Equation 6 [24]. Like the S/N, it reflects the ratio between the noise and the assay window. However, in contrast to S/N, the $Z'$-factor accounts for the deviation in the signal as well as in the baseline, and thus for the likelihood of both false negatives and false positives. To measure the $Z'$-factor, the mean ($\mu$) and SD of positive (subscript C+; maximum signal) and negative control (subscript C−; no signal) have to be determined.

$$Z' = 1 - \left(\frac{3SD_{C+} + 3SD_{C-}}{|\mu C + - \mu C -|}\right)$$

Equation 6

With the adjustments discussed in Chapter 2.1.2.a, we derived the $Z'$-factor for HRS in Equation 7 [Chapter 2.2]. $\mu_c - \mu_{c,max}$ is identified with $\mu_{c,min}$ and consequently, $SD_c$ changes to $SD_{c,min}$. As discussed, the baseline represents the internal (negative) control and therefore $SD_c$ becomes the noise.

$$Z_{chrom} = 1 - \left(\frac{3SD_{Hmax} + 3\text{noise}}{|H_{max}|}\right)$$

Equation 7

HRS platforms have been successfully used to quantify the affinity/activity of analytes by measuring their IC$_{50}$ values. However, this is no core strength of HRS platforms as to this end they still rely on purified compounds as much as HTS platforms, due to their still missing capacity of HRS platforms to quantify unknown compounds (see Chapter 5). Nonetheless, it is valuable to discuss the measurement of IC$_{50}$ values in HRS platforms. In the context of validation, good sigmoidal curves confirm that signal changes observed are indeed due to interaction of the analyte with the target and do not result from (non-specific) artifacts or variations in the assay. If literature values measured with the same assay principle are available, the actual IC$_{50}$ values provide a validation. However, great caution is advised when comparing IC$_{50}$ values as there are many pitfalls. Even seemingly identical assay formats can show major differences. A classic example is the use of different substrates in an activity assay, but sometimes changes in buffer constituents might impact the interaction of analyte and target as well. Additionally, kinetics might impact IC$_{50}$ values when comparing assays with largely different incubation times (see Chapter 2.1.2.a). Influence of shorter incubation times) [Chapter 2.2] [18]. However, this is by no means an issue unique to HRS, although it appears more pronounced than in HTS. In terms of application, measuring IC$_{50}$ values with HRS sometimes offers an advantage as well. Due to the influences discussed in The assay signal [Chapter 2.1.2.a]), HRS assays are often either of higher quality at the same protein concentration or necessitate a lower protein concentration to achieve the same quality. While the influence of the former on IC$_{50}$ value quality is evident, the latter allows a better distinction of high-affinity binder/inhibitors [Chapter 2.2. The reason is that half of the protein concentration (c$_c$) is the theoretical lower limit of measurable IC$_{50}$ values, because below that limit the enzyme is simply titrated. For example, we have c$_c$ of 90 and 450 nM in an HRS assay and in an HTS assay, respectively, and an analyte with an IC$_{50}$ of 45 nM. If we add the analyte at 45 nM, it interacts with 50% of the protein in the HRS assay thus correctly giving an IC$_{50}$ value of 45 nM. Note that also all analytes with higher affinity yield this value. If we add the same analyte concentration to the HTS assay, it only occupies 10% of the protein binding sites. Only with 225 nM of the analyte, we reach 50% interaction thus the apparent IC$_{50}$ in the HTS is 225 nM (0.5•c$_c$). We should add that this advantage is more often observed in affinity assays as in activity assays the c$_c$ can be reduced by increasing the incubation time.

Similar to HTS, IC$_{50}$ values are determined by measuring a series of concentrations and...
fitting the resulting dose-response curve. To this end, the individual concentrations of the pure compound are injected into the HRS platform. This can be done either in flow injection analysis (FIA) mode accounting for the injection peak by subtraction of a blank injection or by isocratic elution with minimal retention thus separating the injection peak. Then, the critical step is the calculation of $c_i$ by dividing the injected concentration $c_i$ by the two dilution events (see Equation 5). The models and algorithms used for the dose-response curve and calculating the IC50 are the same as in HTS. Theoretically, it is possible to deduce individual IC50 values by injecting a dilution series of a mixture. The repeatability of IC50 values in HRS systems seems to be quite good, as can be judged by almost identical values obtained for TAK-715 in different studies (81 nM (71 to 92 nM) [Chapter 2.2] and 71 nM (36 to 140 nM) [Chapter 3.2]).

A good approach to test the complete HRS platform, and especially the structure-affinity correlation, is the analysis of mixtures of pure compounds, preferably containing both effectors (inhibitors, ligands, etc.) and non-binders [Chapter 2.2]. The first indication is that the correct number of compounds is detected in the MS response and that only as many signals as effectors are detected in the bioassay response. Then, the MS and bioassay responses are matched blindly and the compounds are identified from the MS response. Only the effectors should show bioaffinity.

2.1.3 Conclusions

The strong link between molecular structure and individual biological effect, which is created by the integration of separation, bioassay and structure elucidation, is undoubtedly the greatest potential of HRS. In addition, the (virtually) closed system, the efficient mixing and heat exchange, and the internal control offered by the baseline hold the possibility to achieve high-quality bioassays in the on-line post-column mode. Though throughput is not a primary attribute of HRS, the data density intrinsic to the approach is so high that it presents both a potential and a challenge. Similar to any hyphenated system, a challenge derived concepts would surely benefit from further refinement. Exploring the potentials of HRS can certainly lead to routine application in the future, especially if accompanied by continued technical development of the systems.

References

Chapter 2.2

Development of an on-line p38α mitogen-activated protein kinase binding assay and integration of LC–HR-MS

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Abstract

A high resolution screening method was developed for the p38α mitogen-activated protein kinase to detect and identify small molecule binders. Its central role in inflammatory diseases makes this enzyme a very important drug target. The setup integrates separation by high performance liquid chromatography with two parallel detection techniques. High resolution mass spectrometry gives structural information to identify small molecules while an on-line enzyme binding detection method provides data on p38α binding. The separation step allows the individual assessment of compounds in a mixture and links affinity based methods to pharmacology via the retention time. Enzyme binding was achieved with a competitive binding assay based on fluorescence enhancement which has a simple principle, is inexpensive and easy to interpret. The concentrations of p38α and the fluorescence tracer SKF&B6002 were optimized as well as incubation temperature, formic acid content of the LC eluents and the material of the incubation tubing. The latter notably improved the screening of highly lipophilic compounds. For optimization and validation purposes, the known kinase inhibitors BIRB796, TAK715 and MAPK11 were used among others [14, 15]. These formats require expensive reagents (antibodies, etc.) for activity based methods, scintillation [11] and fluorescence polarization [16] have been used among others. The result is a high quality assay with Z' factors around 0.8, which is suitable for semi-quantitative affinity measurements and applicable to various binding modes. Furthermore, the integrated approach gives affinity data on individual compounds instead of averaged ones for mixtures.

Introduction

Kinases are certainly among the most important target classes in contemporary drug discovery [1]. The mitogen-activated protein kinase p38 (p38) is one prominent example of a drug-target kinase especially its most important isoform p38α [2, 3]. It is involved in cell proliferation, extracellular signal regulated pathways, transmitting signals through successive activation steps by phosphorylation [4]. Being one of the important mediators for a cell’s response to extracellular stimuli, this pathway presents a promising opportunity for drug intervention [5]. Because p38α is heavily involved in inflammation processes, the envisaged diseases are mostly chronic inflammatory diseases like rheumatoid arthritis, psoriasis or Crohn’s disease [6]. Several p38α inhibitors, which target these diseases, are currently in different stages of preclinical and clinical development up to phase II [7, 3]. Nearly all known kinase inhibitors show interactions with the highly conserved ATP-binding pocket. Therefore, most early kinase inhibitors, possessing only these interactions, exhibited poor kinase selectivity [5]. Although different systems of categorization exist, these will be called type I inhibitors. Type I inhibitors used in this study are TAK715 [8], MAP kinase inhibitor 1 (MAPK11) from Merck KGaA [9] and SKF&B6002 (SKF) [9]. The discovery of an allosteric binding pocket adjacent to the ATP-binding site led to the discovery of inhibitors using both sites (type II). BIRB796 is a prominent example of this type of binding [10, 11]. Consequently, type III binders like 1-(3-tert-buty1-1-methyl-1H-pyrazol-5-yl)-3-(4-chlorophenyl)urea (pyrazolourea) are targeting only this allosteric site [12]. The type IV inhibitors are not binding to either of the two pockets, as has been discovered e.g. for GNF-2 binding to the Brc-abl kinase [13]. The importance of p38α has led to a multitude of assay principles for the identification of small molecules targeting this kinase [14, 15]. Among the cell-free in vitro formats for p38α, those based on activity and those based on affinity can be distinguished. As readout for activity based methods, scintillation [11] and fluorescence polarization [16] have been used very commonly by others [14, 15]. These formats require expensive reagents (antibodies, etc.) and rely on labels which can impair the usefulness of results, or they involve handling radioactivity. In addition to these drawbacks, activity assays can, by definition, not employ the non-phosphorylated kinase as target. This is the advantage of affinity based assays. However, scintillation [17], chemiluminescence [16] and fluorescence polarization [18] suffer from similar reagent based drawbacks as mentioned above. Furthermore, some label free technologies, like surface plasmon resonance [16] or isothermal titration calorimetry [19], have been employed, which avoid additional reagents and the problems of molecular probes. Still, these techniques are complex and instrumental requirements and logistics may limit their throughput. A relatively simple approach was used by Pargellis et al during the discovery of BIRB796 [10, 11]. The method is based on fluorescence enhancement and requires solely native p38 and the small molecule kinase inhibitor SKF. SKF shows fluorescence, which is strongly enhanced upon binding to the target enzyme. Therefore, high fluorescence intensity indicates a high concentration of the enzyme-tracer complex. A compound showing affinity to the target enzyme will compete with the tracer thus reducing the concentration of enzyme-tracer complex and thereby reducing the fluorescence. This principle enables a competitive binding assay in a homogeneous setup, which is based on the strong difference in fluorescence intensity between the free tracer and the enzyme-tracer complex. Because of its convenience and the possibility to use non-phosphorylated p38α as target, this technique presented a good basis for the development of a high resolution screening (HRS) platform. HRS is defined by the combination of separation and screening in an integrated system. Therefore, it enables the affinity assessment and the simultaneous structural characterization of individual compounds in mixtures [20, 21]. This feature makes HRS platforms especially attractive when complex mixtures such as metabolic incubations [22], combinatorial mixtures [21] or natural extracts [23] are screened where dereplication, affinity and structural information are crucial. In contrast to conventional high throughput screening (HTS), isolation of individual compounds not showing affinity can be avoided. Thus, the laborious development of high yield synthesis and isolation methods can be limited to compounds showing the desired affinity. One common approach in HRS is the use of on-line post-column assays, which have been recently reviewed by Shi et al [24]. Combining fluorescence and mass spectrometric readout, compounds were detected in both homogeneous and inhomogeneous formats. Homogeneous setups are easier to apply, so their significance exceeds inhomogeneous setups in cases where background fluorescence is of minor importance. However, HRS formats described so far are either activity based [21, 23] or target a receptor [20, 22]. We report the implementation of the enzyme system for the first time as an on-line post-column system integrated in an HRS system. Furthermore, the screening of highly lipophilic molecules was greatly improved by the unprecedented use of covalently modified fused silica tubing as material for post-column incubation, resulting in improved affinity signals.

Materials and Methods

Abstract

Human recombinant p38α, ORGX, BIRB796 and TAK715 were a kind gift of MSD research laboratories (Oss, The Netherlands). SKF and MAPK11 were purchased from Merck KGaA (Darmstadt, Germany). ELISA blocking reagent was delivered by Roche Diagnostics (Mannheim, Germany). Pyrazolourea was obtained from Ambiber (Paris, France). All other chemicals were from Sigma-Aldrich (Schnelldorf, Germany). Fused silica tubing (250 μm inner and 375 μm outer diameter) untreated or covalently coated with PEG, methyl or methyl/phenyl groups was also purchased from Sigma-Aldrich (Schnelldorf, Germany). 96 ‘flat’ bottom chimney well, black polypropylene microtiter plates from Greiner bio-one (Alsbach, Germany). The Netherlands) were used. Methanol (LC-MS Grade) and formic acid (UPLC-MS Grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Purified water was generated with a Milli-Q academic from Millipore (Amsterdam, The Netherlands).

Instrumentation

96 well plate experiments were performed on a Victor3 plate reader from Perkin-Elmer (Groningen, The Netherlands). The setup used for the on-line assay included two LC-20AD and two LC-10AD isotropic pumps, a SIL-20AC autosampler, one CTO-20AC and one CTO-10AC column oven, an RF-10AXL fluorescence detector, a CBM-20A controller and
an Ion Trap-Time-of-flight (IT-TOF) mass spectrometer equipped with an ESI source all products of Shimadzu (‘s Hertogenbosch, The Netherlands). HR-MS spectra were obtained from m/z 200 to 650 switching positive- and negative-ion mode with 10 ms accumulation time. The measured desolvation line and the heating block were equilibrated at 200°C and the voltages applied were 5 kV to the interface and 1.7 kV to the detector. Nitrogen (99.9990%) was used at flows of 1.5 L/min and 10 L/min as nebulising gas and drying gas, respectively. To allow accurate mass measurements, external calibration of the IT-TOF was performed using TFA clusters.

Furthermore, two superloops and two pulse dampeners made in house were used.

**Plate Reader Assay**

A 20 mM TRIS-HCl buffer (pH 7.5), containing 10 mM MgCl₂, 2 g/L PEG 6000 and 0.2 g/L ELISA blocking reagent (TRIS buffer), was used in all experiments. All solutions of p38α were prepared in TRIS buffer. Enzyme stock solutions were stored at –40°C until use. During on-line experiments, the enzyme solutions were kept at 0°C. A stock solution of SKF (2 mM) was made in DMSO and further diluted with TRIS buffer. Solutions of inhibitors were prepared in water:methanol 1:1 containing 0.01% formic acid from 2 mM or 5 mM stock solutions in methanol (DMSO in case of MAPKI1). Small molecules stock solutions were stored at -20°C.

For plate reader measurements, the wells were filled with 50 μL enzyme solution, 12.5 μL inhibitor solution and 50 μL SKF solution in the given order. Fluorescence was measured at 25°C with the wavelength of absorbance and emission being 355±4 nm and 405±5 nm, respectively. The sum of ten flashes was used as readout to improve performance. Saturation of a 90 nM solution of p38z with SKF was determined by using a concentration range from 0 to 6000 nM of SKF. The z-factor is a measure for assay quality. If not otherwise stated, it was calculated according to the original publication of Zhang et al [25].

A more detailed explanation is given in the results and discussion section. Measurements of the z-factor dependence on enzyme concentration were performed on three days with five repeats per day and a final concentration of 3 μM SKF. For full inhibition, TAK715 was added to the other concentration of 1 μM, while the control values contained only the corresponding amount (see above) of HPLC solvents.

**On-line Assay**

HPLC solvents were water:methanol 100:1 (solvent A) and methanol:water 100:1 (solvent B) both containing 0.01% formic acid. The instrumental setup described by de Vlieger et al [20] was used for the analysis with one enzyme binding detection line and the following modifications (cf Figure 1). HPLC was done either in flow injection analysis mode (FIA – where no separation column is present) or with a SunFire C18 column 30 x 2.1 mm, 3.5 μm particles (Waters, Milford, MA, USA) (Figure 1 - 2). 10 μL of the sample were injected (Figure 1 - 1) into a flow of 113 μL/min. Elution was performed at 40°C either isocratic at different methanol concentrations or using a binary gradient. Post-column, the flow was split 1:9 (Figure 1 - 3), sending 100 μL/min to high resolution/mass spectrometry (HR-MS) detection and 13 μL/min to the enzyme binding detection. To the 13 μL/min HPLC eluent, first 50 μL/min of a 200 nM p38α solution were added (Figure 1 - 4) and thereafter 50 μL/min of a 1400 nM solution of SKF (Figure 1 - 6). These solutions were constantly released by a linear setup of an LC-10AD pump, a pulse dampener with restriction capillary and a superloop each. Following each addition, the mixture was incubated in PEG deactivated fused silica loops of 25 μL (Figure 1 - 5) and 20 μL (Figure 1 - 7), respectively. The incubation loops were kept at 25°C in a second column oven. The resulting incubation times were 24 s for the target-ligand interaction (Figure 1 - 5) and 11 s for the target-tracer interaction (Figure 1 - 7). Finally, at concentrations of 90 nM p38α and 630 nM of SKF the fluorescence was measured with excitation at 355±7.5 nm and emission at 425±7.5 nm (Figure 1 - 8).

The used detector settings were gain 4, recorder range 1 and sensitivity medium. The influence of the methanol concentration on the maximum signal was assessed in two consecutive measurements. To this end, a stepwise gradient was used starting at 100% A and increasing the fraction of B by 15% approximately every 10 min. In the first run, the baseline without inhibition was recorded. In the second run, MAPKI1 was added to the SKF solution for full inhibition, reaching a final assay concentration of around 0.9 μM. By comparing these measurements, the assay window in relation to the different organic modifier concentrations can be estimated.

In order to assess the influence of the incubation tubing material, two measurements were carried out, in which the volume and therefore the incubation time was kept constant: In the first experiment, polyether ether ketone (PEEK) of 250 μm and 170 μm inner diameter (ID), polytetrafluoroethylene (PTFE) of 250 μm ID and untreated fused silica of 250 μm ID were compared by injecting a 2 μM solution of ORGX in FIA mode at 60% B. In a second experiment, 250 μm ID fused silica tubing with covalent coatings of different polarity were compared. In these experiments, a 20 μM solution of ORGX was injected in FIA mode at 60% B. The coatings were PEG (polar), a mixture of phenyl and methyl groups (intermediate) or methyl groups only (non-polar).

The on-line z-factor was calculated from triplicate injections each of a control (blank) experiment and a full inhibition sample on five different days. The full inhibition sample was a mixture of 50 μM BIRB796, 20 μM ORGX and 20 μM TAK715 while the control sample was a 1:1 mixture of the solvents. The separation was carried out with the following gradient: 0 to 1.0 min isocratic at 50% B, 1.0 to 12.5 min linear to 90% B, 12.5 to 14.5 min isocratic at 90% B, 14.5 to 15.0 min linear to 50% B and 15.0 to 21.0 min isocratic at 50% B. MAPKI1 and TAK715 were tested in a mixture with six drug molecules to simulate a screening situation. Carbamazepine, norethisterone, warfarin, phenylbutazone, celecoxib and diclofenac were used as test compounds without p38α affinity. TAK 715 was injected at 20 μM concentration, the others at 50 μM. The mixture was separated for the following gradient: 0 to 2.0 min isocratic at 50% B, 2.0 to 32.0 min linear to 70% B, 32.0 to 38.0 min isocratic at 70% B, 38.0 to 38.5 min linear to 50% B and 38.5 to 50.0 min isocratic at 50% B.

For the measurements of IC₅₀ values, isocratic elution was applied, taking care that the substance peak showed separation from the injection peak. TAK715 was eluted at 85% B, BIRB796 at 50% and MAPKI1 at 70%. Solutions of nine different concentrations for each inhibitor were prepared in 50% methanol, 50% water and 0.01% formic acid from 5 mM stock solutions of TAK715 and BIRB796 in methanol or a 2 mM stock solution of MAPKI1 in DMSO. The following concentrations for each inhibitor were prepared: 50 nM, 100 nM,
200 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM and 20 μM for TAK15; 500 nM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM 50 μM, 100 μM and 200 μM for BIRB796 and 200 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM 50 μM and 100 μM for MAPK11. These nine concentrations and a blank were injected in triplicate for every inhibitor.

To finalise the binding mode studies, the pyrazolourea compound was used as an example of a type III inhibitor. It was injected at a concentration of 100 μM and tested under isocratic conditions at 70% B.

Results and Discussion

Assay conditions optimization

Optimization was carried out in a 96 well format. The protocol of Regan et al [10] was used with some modifications. Since Mg²⁺ ions are known to be involved in ATP binding and can potentially influence binding of inhibitors to the ATP binding site, MgCl₂ was added. Furthermore, ELISA blocking reagent and PEG 6000 were added in order to avoid non-specific binding and unwanted interactions with the material of the enzyme binding detection [26]. Enzyme and tracer concentrations were optimized under these conditions. The fluorescence signal showed a linear dependence on the enzyme concentration (cf Online Resource 1 Figure S1). The most suitable p38α concentration was therefore chosen considering the Z’-factor measurements. The signal dependence on the tracer concentration, depicted in Figure 2A, displays the asymptotic form of saturation behaviour. The final concentration of 630 nM SKF is selected to be slightly above the saturation value. This choice presents a compromise between avoiding deviations of the signal due to variability in the tracer concentration and maximising the sensitivity with regard to detection limits for binders. Namely, competition reduces this sensitivity with increasing tracer concentration.

There are several factors mainly related to the HPLC separation system, which can have a negative influence on the assay performance. Methanol, formic acid and the incubation temperature are the most prominent of these variables. Using typical HPLC conditions such as 50% methanol and 0.1% formic acid at 37°C completely inhibited the fluorescence enhancement. As a result, these factors were carefully optimized and need to be controlled cautiously. The influence of organic modifier at no and full inhibition is shown in Figure 2B. The assay window decreased with increasing organic modifier (methanol) content. However, the Z’-factors for the different compounds measured at different MeOH concentrations, due to gradient elution, showed only minor differences thereby proving the robustness of the on-line setup even at high methanol concentrations. Increasing temperature also yielded a similar decrease in maximum signal as seen with methanol. In order to guarantee robustness and enable the use of up to 90% methanol in the HPLC separation, the formic acid concentration in the eluent and the incubation temperature were restricted to 0.01% and 25°C, respectively. Hence, the temperatures of the HPLC separation step and the enzyme binding detection were controlled separately.

The screening of highly lipophilic compounds is not only challenging in off-line screening due to adhesion and solubility issues [27] but also presents challenges in on-line screening approaches [28]. In on-line screening systems, the amount of organic modifier is usually kept low to prevent protein degradation. On the other hand, the tubing used for incubation loops is mostly PEEK or PTFE. The favoured interaction of non-polar compounds with these surfaces results in peak broadening and tailing, thus decreasing resolution and sensitivity. By using polar material for the incubation loops, this effect was significantly decreased. Figure 3A shows a two to threefold increase in signal height and reduced tailing when untreated fused silica tubing is used instead of PEEK. Although, PTFE compared to PEEK already yields decreased peak width, the width is additionally approximately halved with untreated fused silica. The polarity of the incubation coil material plays a crucial role explaining these findings. This can clearly be seen in Figure 3B where the influence of fused silica tubing with covalent coatings of different polarities on the peak shape was compared. The polar (PEG coated) tubing gave the best results while peak height and shape degraded towards intermediate (methyl/phenyl coated) and non-polar (methyl coated) surfaces. This indicates the influence of tubing polarity on post-column band broadening and justifies a preference of polar coated fused silica tubing over PEEK and PTFE in the presented study, taking the structural features of the studied compounds into account.

Validation of the p38α binding assay

The quality of the p38α binding assay was assessed using the method of Zhang et al [25]. The Z’-factor is introduced as a single value to judge assay quality, because it incorporates the SD of signal and background as well as the assay window. Therefore, it indicates the ability to distinguish between signals and false positives or false negatives. According to this method, the Z’-factor was calculated with equation 1 for several enzyme concentrations in the plate reader based setup. μs and μb are defined as the average signal of the sample and the control, respectively. Consequently, σs and σb are the standard deviations (SD) of these values.

\[
Z' = 1 - \frac{3(\sigma_s + \sigma_b)}{|\mu_s - \mu_b|}
\]

Equation 1

Figure 2: Optimization of the p38α affinity assay. A) Saturation curve of the tracer for 90 nM p38α (n=3). Full saturation was reached at 630 nM SKF, which was consequently the selected tracer concentration. B) Influence of the organic modifier concentration on the enzyme binding detection. I) is the baseline at different percentages of organic modifier in HPLC mobile phase, which shows the maximum signal reached at these different MeOH concentrations. In opposition, line II) shows the fluorescence signal at maximum inhibition hence the difference between both lines describes the asymptotic form of saturation behaviour. The final concentration of 630 nM SKF is selected to be slightly above the saturation value. This choice presents a compromise between avoiding deviations of the signal due to variability in the tracer concentration and maximising the sensitivity with regard to detection limits for binders. Namely, competition reduces this sensitivity with increasing tracer concentration.

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In the plate reader experiments the concentration for this purpose. In the on-line setup ORGX and TAK715 were injected at 20 μM concentration and BIRB796 at 50 μM concentration for this purpose.

Furthermore, the separation step introduces an additional dilution due to chromatographic peak broadening, which requires higher analyte concentrations. There are additional differences between the two setups, which should be adequately reflected in the determination of Z'-factor.

For the sake of comparability, the number of experiments (n=15) was kept the same in plate reader and on-line based measurements. However, this is recognized as a relatively low number for plate reader experiments. Therefore, an additional plate reader experiment with n=50 was performed, yielding a Z’-factor of -0.02. The conclusion from both experiments was as follows: The plate reader based assay at 90 μM p38α gave insufficient results with Z’-factors lower than 0. In the plate reader assay, a Z’-factor above 0.5, indicating an excellent assay, could only be achieved by raising the p38α concentration to 450 nM.

Table 1 compares this data with the results of the on-line setup, in which 90 μM p38α was sufficient for an excellent assay. As a confirmation, the signal to noise ratios (S/N) were also calculated (Table 1), resulting in the same conclusions.

Regardless of the findings of the last paragraph, the on-line setup has two disadvantages. Reagent consumption is usually higher in an on-line setup than in a plate reader based assay. For the sake of comparability, the number of experiments (n=15) was kept the same in plate reader and on-line based measurements. However, this is recognized as a relatively low number for plate reader experiments. Therefore, an additional plate reader experiment with n=50 was performed, yielding a Z’-factor of -0.02. The conclusion from both experiments was as follows: The plate reader based assay at 90 μM p38α gave insufficient results with Z’-factors lower than 0. In the plate reader assay, a Z’-factor above 0.5, indicating an excellent assay, could only be achieved by raising the p38α concentration to 450 nM.

Table 1: Z’-factors and signal to noise ratios (S/N) for plate reader and/or on-line based enzyme binding detection

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>μ [p38α]</th>
<th>Z’ or Z’_{chrom}</th>
<th>n=15</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate reader</td>
<td></td>
<td></td>
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<tr>
<td>TAK715</td>
<td>90</td>
<td>-0.41</td>
<td>4</td>
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</tr>
<tr>
<td>180</td>
<td>0.49</td>
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<td>450</td>
<td>0.75</td>
<td>48</td>
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<tr>
<td>On-line</td>
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</tr>
<tr>
<td>BIRB796</td>
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<td>70</td>
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<tr>
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</tr>
<tr>
<td>TAK715</td>
<td>90</td>
<td>0.80</td>
<td>97</td>
<td></td>
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</table>

*Concentration of the inhibitor was 1 μM TAK715 (final concentration) in the plate reader experiments. In the on-line setup ORGX and TAK715 were injected at 20 μM concentration and BIRB796 at 50 μM concentration for this purpose.

In the plate reader experiments the Z’-factor was calculated according to equation 1. For the on-line experiments, equation 2 was used, yielding the Z’_{chrom}-factor.

\[ Z'_{chrom} = 1 - \frac{3 \cdot SD_h + 3 \cdot noise}{\mu_f} \]

Equation 2

Figure 3: Influence of the reaction coil material on the peak shape. A) PEEK and PTFE were compared with untreated fused silica tubing. A 2 μM solution of ORGX was injected in FIA mode at 60% methanol. B) At the same conditions, a 20 μM solution of the same inhibitor was injected. Fused silica tubing with covalent coatings of either PEG (polar), a mixture of methyl and phenyl groups (intermediate) or only methyl groups (non-polar) was compared for their influence. In both experiments, peak width and asymmetry increased with decreasing polarity while the peak height decreased as a consequence. Fused silica tubing, coated with PEG (polar), exhibited drastically improved performance over the compared materials.

For a complete consideration of the differences between the on-line and the plate reader setup, all classical variables are systematically substituted with parameters more suitable to the on-line approach. Doing this without compromising the comparability of this Z’-factor is not trivial but possible. The assay window (μ_s, μ_f), as determined by the difference of the mean of control (no inhibition) and signal (full inhibition), could more suitably be expressed as the mean of the peak height of full inhibition (μ_f), thereby still describing the desired parameter. Thus, the assay window is automatically corrected for changes in the baseline, representing the internal control. The SD of the sample (σ_s) would then consequentially be identified with the SD of the peak heights at full inhibition. For the control, usually no signals are obtained and changes of the baseline between samples are irrelevant as they have already been taken into account. The SD of the control (σ_f) can therefore logically be identified with the baseline deviation within one sample. This value is in chromatography better known as the noise. The measure of three times the noise is often used as limit of detection. This is consistent with the ideas behind the Z’-factor as both interpretations of 3σ_s are aimed at avoiding false positives. In summary, the transition from an external control to an internal one strengthens the reproducibility and accuracy of the assay and should therefore be appropriately reflected in the determination of Z’-factors. Therefore, an adjusted Z’-factor for the use in on-line systems is proposed in equation 2 on basis of the previous argumentation.

Figure 4: Comparison of MS and enzyme binding detection chromatogram. The matching of the fluorescence trace of the enzyme binding detection (above) and the total ion current (TIC) of the MS signal are obtained and changes of the baseline between samples are irrelevant as they have already been taken into account. The SD of the control (σ_f) can therefore logically be identified with the baseline deviation within one sample. This value is in chromatography better known as the noise. The measure of three times the noise is often used as limit of detection. This is consistent with the ideas behind the Z’-factor as both interpretations of 3σ_s are aimed at avoiding false positives. In summary, the transition from an external control to an internal one strengthens the reproducibility and accuracy of the assay and should therefore be appropriately reflected in the determination of Z’-factors. Therefore, an adjusted Z’-factor for the use in on-line systems is proposed in equation 2 on basis of the previous argumentation.

\[ Z'_{chrom} = 1 - \frac{3 \cdot SD_h + 3 \cdot noise}{\mu_f} \]

Equation 2

Kool et al. already applied the Z’-factor to chromatography based on-line assays, adapting the variables of equation 1 in order to take into account these discrepancies [28]. A main advantage is the internal control value (μ_f) represented by the baseline, which is more relevant than an external control, because it is available for each individual experiment.
The new interpretation of the $Z'$-factor could also be used to omit the measurements of a control, significantly shortening the time of $Z'_{\text{ret}}$-factor determination. Because the noise is calculated from the baseline of the chromatogram, a prerequisite is to have an empty time frame to observe both required values. Therefore, the first 4 min of the enzyme binding detection chromatogram were used to determine the noise. The mean noises calculated from signal and control runs were identical, thus proving the redundancy of the control measurements for $Z'_{\text{ret}}$-factor determinations. 

The repeatability of triplicates is excellent, which is clearly visible in Figure 4, 5 and 6. This is true for the enzyme binding detection responses as well as for the chromatographic parameters like peak width, measured as full width at half maximum (FWHM), and retention time ($t_r$). For the enzyme binding detection, the relative SDs are ≤10% for FWHM and <3% for $t_r$. Low variability and noise compared to the total signal results in the high $Z'$-factor and signal-to-noise ratio (S/N) shown in Table 1. The high $Z'$-factors indicate the excellent intra- and interday reproducibility. However, the on-line setup showed comparable signals-to-noise ratio (S/N) shown in Table 1. The high $Z'$-factors at five times lower enzyme concentration than needed in the plate based assay. This is directly related to the minimal measurable IC$_{50}$. While IC$_{50}$ values down to 45 nM can be distinguished in the on-line method, the plate reader method only allows differentiation of IC$_{50}$ values of 230 nM and higher. Furthermore, the higher enzyme concentration needed diminishes the advantage of the plate setup with regard to reagent consumption. Therefore, the on-line setup has advantages beside the additional possibilities associated with separation.

**High resolution screening of mixtures**

In order to assess whether the presented HRS method was suitable for the identification of small molecule binders of p38α in mixtures, a solution of eight compounds was screened. Two of these were the kinase inhibitors MAPKI1 and TAK715 while the other six were drug molecules from different classes. As illustrated in Figure 5, all eight compounds could be separated for individual identification and affinity assessment. Based on HR-MS data, the structure was linked to a retention time. Accurate mass measurements enabled the unambiguous distinction of all compounds as two of them differed by less than 0.05 u. In case of more scarce knowledge of the sample, structure elucidation can be facilitated by HR-MS$^+$ experiments [21]. In parallel to the structure confirmation, the retention time is also linked with affinity information through the enzyme binding detection. However, the readout of the enzyme binding detection is delayed compared to the HR-MS readout, because of a higher void volume and a lower average flow rate after splitting. This delay was measured to be 0.5 min by comparing both detection times in several experiments. It remains constant as long as void volume and flow rates are unchanged. The retention time corrected for this delay is used to couple affinity to identity information. Two affinity peaks were detected, which were linked to the elution of the kinase inhibitors. In conclusion, the approach presents a fast method to match the structure and activity of compounds in mixtures.

**Semi-Quantitative affinity measurements**

Several known p38α inhibitors were tested for their dose related responses in the enzyme binding detection. Therefore, their injected concentrations had to be converted to final assay concentrations by taking into account the dilution factors. On one hand, splitting of the HPLC eluent and subsequent mixing with the reagents leads to dilution. This can be easily calculated from the ratios of the flow in the beginning and the end of the enzyme binding detection (113/13 = 9). On the other hand, the chromatographic dilution, meaning the transition from an injected plug to a series of peaks of (assumed) Gaussian shape, has to be taken into account. The corresponding calculations have been described elsewhere [29]. The necessary peak parameters were taken from the enzyme binding detection. By dividing the injected concentration by the product of both dilution factors, the final concentration at maximum peak height can be calculated and used in the IC$_{50}$ calculations.

**Figure 5:** HRS of a mixture of kinase inhibitors and drug molecules. MAPKI1 (4) and TAK715 (8) were mixed with carbamazepine (1), norethisterone (2), warfarin (3), phenylbutazone (5), celecoxib (6) and diclofenac (7). The mixture was separated by HPLC and measured with HR-MS and enzyme binding detection in parallel. Accurate mass measurements with low deviation (<8ppm) and well matching isotope patterns allowed unambiguous identification. Structure and affinity were easily matched via the corrected retention times. Consequently, the two affinity compounds were detected in the mixture.

**Figure 6:** Quantitative data of the tested p38α inhibitors. Binding curves are depicted for several known p38α inhibitors. The inhibitors TAK715 (circles), BIRB796 (squares) and MAPKI1 (triangles) are shown. Standard deviations of triplicate measurements were smaller than data point size.
Dose-response curves depicted in Figure 6 were measured in triplicate with excellent R²-values of >0.998 and minimal standard deviation (SD) of the triplicates (<2% SD relative to assay window). This shows again the excellent reproducibility and accuracy of the assay. The IC₅₀ values and, given in brackets, their 95% confidence intervals, calculated after correcting the concentrations for the dilution factors are 81 nM (71 to 92 nM) for TAK715, 400 nM (360 to 440 nM) for BIRB796 and 760 nM (530 to 1100 nM) for MAPKI1. If the inhibitors are ranked for their affinity, the obtained results are in agreement with other methods, stating values of 7.1 nM for TAK715 [8], 18 nM for BIRB796 [10] and 35 nM for MAPKI1 [9]. However, the absolute values of measurements are 10 to 20 times higher than in literature. This can be explained by the profound differences in the assay formats. While the reported method gives affinities, the reference values were obtained by either activity assays (TAK715) or cell based functional assays (BIRB796, MAPKI1). Another specific reason responsible for this deviation is the slow binding kinetics of BIRB796. This compound is known to show an up to 12-fold deviation of its IC₅₀ value, depending on the incubation time [11]. Despite the aforementioned issues, the method was proven to be suitable for the semi-quantitative assessment of small molecule relative affinities towards p38α serving as a selection tool in the drug discovery process.

Applicability and binding modes

The type of binding to p38α as discussed in the introduction can have a significant influence on the ability of a screening technology to detect this binding. SKF is a type I binder and should therefore enable the detection of type I and type II binders as they compete for the ATP-binding pocket. This has been proven by the detection of TAK715 and MAPKI1 (type I) as well as BIRB796 (type II). Furthermore, structural changes in p38α upon binding of type III inhibitors may allow the detection of such compounds. The rearrangement of p38α from the DFG-in to the DFG-out conformation results in a reorientation of Phe405 thereby affecting the ATP-binding pocket. Hence, we tested the pyrazoloure structure, which was discussed by Simard et al as an example of a type III inhibitor [12]. Figure 7 shows the enzyme binding detection of this compound to p38α, thereby proving the suitability of the method for the detection of type III inhibitors. Consequently, the assay is capable of detecting a wide range of p38α binders including type I, II and III binders.

An on-line binding assay for the separation and parallel assessment of identity and affinity of small molecules in complex mixtures towards non-phosphorylated p38α was developed. In terms of applicability, the scope of the presented HRS platform is different from conventional HTS assays. HTS is more suited for the screening of huge libraries where speed and low sample consumption are essential. The value of HRS lies in the parallel determination of structural and biochemical data of potential binders. While an HTS assay will only give a sum of compound affinities, HRS gives individual information on all (separated) molecules. The aim was to make available the advantages of the on-line approach, mainly the measurement of mixtures and a comparison of structure and affinity, for the important drug target p38α. An on-line post-column enzyme binding detection based on fluorescence enhancement was developed and its compatibility with modern HPLC separation techniques, such as gradient elution and increased temperature, was demonstrated. As HRS as parallel readout is an integral part of the developed screening platform, allowing the identification of the tested molecules. Additionally, the prospects of measuring compounds individually in mixtures were demonstrated by matching identity and affinity. Regarding the presented assay validation data, it can be stated that a high quality assay was achieved. Finally, the assay provided ranking of various p38α inhibitors (types I, II and III) allowing the use within a drug discovery process.

Acknowledgements

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References


Figure 7: Detection of the binding of the pyrazoloure type III inhibitor to p38α. Triplicate injections of the inhibitor (black) showed signals in the enzyme binding detection (top). In contrast, the control (grey) did not show binding to p38α. HR-MS (bottom) confirmed the pyrazoloure compound as the binder. This clearly shows the ability of the assay to detect type III inhibitors. In combination with Fig. 4 it proves the applicability to several types of p38α inhibitors (types I, II and III).

Supplementary Material

Figure S1: Baseline signal dependence on enzyme concentration in the plate reader setup. The maximum fluorescence, equivalent to the maximum enzyme-tracer complex concentration, was measured at different enzyme concentrations. 1250 nM of SKF were used. The data justifies the homogenous setup as it proves the insignificance of the background fluorescence at the tested concentration. Furthermore, it suggests that the assay is tuneable to a desired compromise between quality and enzyme consumption.

Calculation of final inhibitor concentration

There are two factors of dilution which diminish the concentration of the tested compound in the enzyme binding detection. The first arises from the mixing of the HPLC eluent with the reagents of the enzyme binding detection. It can therefore be calculated using mixing dilution ($D_m$). $D_m$ is easily calculated by dividing the final flow rate in the fluorescence detector ($u_f$) with the flow rate of the HPLC eluent entering the enzyme binding detection ($u_e$), because this represents the ratio of volumes in any given time frame.

$$D_m = \frac{u_f}{u_e}$$

The second factor is the chromatographic dilution ($D_c$). It can be calculated assuming a symmetrical peak of Gaussian shape. Based on this assumption, the ratio between the height of a plug of the injection volume and the resulting Gaussian peak equals the dilution factor. Influences are the full width at half maximum (FWHM), the flowrate in the HPLC ($u_e$) and the injection volume ($V_i$). It is imperative that the FWHM is taken from the fluorescence and not from the MS chromatogram. The bigger void volume in the enzyme binding detection leads to a broader peak because of diffusion and surface interactions.

$$D_c = \frac{FWHM}{2} \sqrt{\frac{\pi}{\ln 2}} \frac{u_e}{V_i}$$

Equation S2 [1]

By combining these two factors the final concentration ($c_f$) of the binders can be determined from the injected concentration ($c_i$).

$$c_f = \frac{c_i}{D_m \cdot D_c}$$

Equation S3 [1]

Chapter 2.3

Development of on-line liquid chromatography-biochemical detection for soluble epoxide hydrolase inhibitors in mixtures

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Abstract

In this study, an end-point based fluorescence assay for soluble epoxide hydrolase (sEH) was transformed into an on-line continuous-flow format. The on-line biochemical detection system (BCD) was coupled on-line to liquid chromatography (LC) to allow mixture analysis. The on-line BCD was based on a flow system wherein sEH activity was detected by competition of analytes with the substrate hydrolysis. The reaction product was measured by fluorescence detection. In parallel to the BCD data, UV and MS data were obtained through post-column splitting of the LC effluent. The buffer system and reagent concentrations were optimized resulting in a stable on-line BCD with a good assay window and good sensitivity (S/N > 60). The potency of known sEH inhibitors (sEHis) obtained by LC–BCD correlates well with published values. The LC–BCD system was applied to test how oxidative microsomal metabolism affects the potency of three sEHis. After incubation with pig liver microsomes, several metabolites of sEHis were characterized by MS, while their individual potencies were measured by BCD. For all compounds tested, active metabolites were observed. The developed method allows for the first time the detection of sEHis in mixtures providing new opportunities in the development of drug candidates.

Introduction

Soluble epoxide hydrolase (sEH) plays an important role in regulation of blood pressure, pain and inflammation [1]. In mammals, sEH is expressed in various tissues. The endogenous substrates of sEH are among others epoxyeicosatrienoic acids (EETs), which are hydrolyzed to dihydroxyeicosatetraenoic acids (DHETs), thus leading to decreased blood levels of EETs. Various studies show, that EETs and epoxides of other unsaturated fatty acids are anti-inflammatory, analgesic agents and lower blood pressure [2; 3; 4]. The biological levels of epoxy fatty acids can be increased by sEH inhibitors (sEHis), leading to reduction in inflammation, pain, and cardiovascular diseases in various animal models [5; 6]. Thus, sEHis are a promising new class of pharmaceutical drug candidates.

During the lead development process, metabolism studies play an important role. Not only the pharmacokinetic profile, but also the biological effects of metabolites are relevant for the action of drugs. Metabolites can be inactive, reactive, but also pharmacologically active towards the same pharmacological target or against off-targets. Screening metabolic mixtures for individual bioactive metabolites is not possible with standard end-point plate-reader based screening methodologies. For sEH, several end-point assays have been developed, based on fluorescent detection, radiometry, and mass spectrometry [7; 8; 9]. However, these assays are only suited to screen pure compounds. The analysis of mixtures would only yield the sum of the total bioactivity of the mixture [10]. In order to assess the bioactivity of individual metabolites, fractionation has to precede the screening. As this is time-consuming, costly, and has to be performed at low resolution to prevent too much dilution, such approaches are inefficient for bioactivity profiling of metabolic mixtures [11]. One way of tackling this problem is the application of an on-line post-column screening approach, known as high-resolution screening (HRS) [12]. This technology continuously mixes bioassay reagents with the eluent after an LC separation of a mixture of compounds [13; 14; 15], such as metabolic mixtures [16]. For HRS screening with enzyme targets, like in this LC–BCD system, inhibition can be measured by detecting a decrease in the enzymatic formation of a fluorescent product. Splitting part of the LC eluent between the BCD and mass spectrometry (MS) enables correlation of bioactivity with identity for all individual metabolites [14; 17; 18].

This paper describes the development of an LC–BCD system for sEHis and its application in the efficient profiling of active oxidative metabolites. For this purpose, a fluorescence end-point plate-reader assay [7] was converted into an on-line BCD format. The on-line BCD uses the non-fluorescent substrate (3-phenyl-oxiranyl)-acetic acid cyanoc-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME), which is converted to the fluorescent product 6-methoxy-2-naphthaldehyde by sEH. The new analytical method was thoroughly optimized and validated. The obtained inhibition efficacy of known inhibitors compared well to literature values. Finally, microsomal incubations of three sEHis were screened for active metabolites.

Materials and Methods

Materials

Human recombinant sEH was expressed and purified as described [19]. The sEH inhibitors and the substrate (3-phenyl-oxiranyl)-acetic acid cyanoc-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME) as well as its fluorescent product are shown in Figure 1. Their synthesis was reported earlier: PHOME [8]; sEHi 1 [8]; sEHi 2 [20]; sEHi 3 [21]; sEHi 4 [22]; sEHi 5 [23]; sEHi 6 and sEHi 7 [24]. ELISA blocking reagent (EBR) was purchased from Roche Diagnostics (Mannheim, Germany). All other chemicals were from Sigma-Aldrich (Schnelldorf, Germany). Methanol (LC–MS Grade) and formic acid (ULC–MS Grade) were obtained from Biosolve (Valkenswaard, The Netherlands). The water used in this study was generated with a Milli-Q academic from Millipore (Amsterdam, The Netherlands).

Liquid chromatography – biochemical detection (LC–BCD) system

The LC–BCD system shown in Figure 2 includes separation, on-line BCD and additional parallel spectrometric detection. Separation and on-line BCD were done on an Agilent Technologies (Amstelveen, The Netherlands) 1100 LC system including a binary and two isocratic pumps, an autosampler, a column oven and a fluorescence detector. Separation...
was performed on a Waters (Milford, MA, USA) Atlantis dC18 column (100 x 2.1 mm, 3 μm particles) at 40°C. The mobile phase consisted of water:methanol:acetic acid 100:1:1 (v:v:v) as solvent A and water:methanol:acetic acid 100:1:1 (v:v:v) as solvent B, both containing 0.01% formic acid. A mixture of known sEHis (125 μM each) was separated using the following gradient: 0 to 2 min isocratic at 50% B, then a linear gradient to 90% B in 43 min and isocratic at 90% B for 4 min; afterwards a linear decrease to 50% B in 2 min followed by 15 min re-equilibration. The LC gradient applied for the analysis of the metabolic incubations was as follows: 0 to 2 min isocratic at 5% B, followed by a linear gradient to 95% B in 43 min, isocratic at 95% B until 49 min then a linear decrease to 5% B in 6 min followed by 10 min re-equilibration. For analysis in flow injection analysis mode (FIA), the same set-up was used, but without the column. The LC flow-rate was 150 μL/min and the injection volume 10 μL in all cases. The flow was post-column split (see Figure 2), directing 135 μL/min to the MS detection and 15 μL/min to the on-line BCD. In the BCD, the eluent was first mixed with 155 μL/min of a 5 nM sEH solution and incubated for 30 s. This allowed an initial interaction between the analytes and enzyme. In a second step, a 30 μM solution of PHOME was added at 30 μL/min and incubated for 5 min. Here, the substrate conversion to the fluorescent product took place which allowed detection of the enzyme activity. The incubations were done in 1.73 m straight 250 μm i.d. and 1.59 mm o.d. PTFE tubing (Sigma-Aldrich, Schnelldorf, Germany) and 2.28 m of 750 μm i.d. and 1.59 mm c.d. coiled PTFE tubing, forming 85 μL/min. Both reactors were kept at 37°C by a Grant Instruments (Shepreth, UK) water bath. The enzyme and substrate solutions were delivered by in-house built superloops which were kept on ice [25]. The fluorescence was measured at excitation of 320±10 nm and emission of 460±10 nm. In parallel to the on-line BCD, detection was performed by UV at 210 nm and/or by mass spectrometry. Both techniques are able to visualize both the binders and the non-binders. Additionally, MS provides structural information. The on-line BCD and the parallel UV or MS detection have different void volumes after the splitting and thus the elution times differ. The UV or MS detection and subsequent BCD chromatograms were aligned using a known compound, e.g., the residual parent compound in case of the metabolic incubations.

Figure 2: Setup of the LC–BCD system. The system combines separation, on-line BCD and additional UV or MS detection in parallel. It includes (1) autoinjector, (2) reversed-phase LC column, (3) flow-splitting between parallel (9) UV or ES-MS detection and (4–8) the on-line BCD. The BCD comprises of (4) mixing of LC effluent and an sEH solution, (5) incubation with the enzyme, followed by (6) mixing of PHOME solution, (7) incubation with PHOME, and finally (8) fluorescence detection.
bottom chimney well, polypropylene microtiter plates from Greiner bio-one (Alphen a/d Rijn, The Netherlands) were used. The total sample volume was 200 μL and the plates were incubated at 37°C. Product formation was followed by measuring the fluorescence at 355 ± 4 nm excitation and 460 ± 12.5 nm emission. The PHOME concentration was 50 μM and the sEH concentration 40 nM. Product formation was measured in 30 s intervals for 20 min under the influence of two different BSA concentrations, 0.1 g/L and 1.0 g/L. End-point measurements at 6 min were used to compare the activity of sEH under the influence of several solubilising agents. In additional experiments, the solubility of PHOME under the influence of these solubilising agents was tested in transparent plates. This was done by measuring precipitation of a 45 μM PHOME solution by visible absorption at 595±10 nm.

**Microsomal incubations**

The LC-BCD/MS system was applied to investigate the metabolism of the three known sEH inhibitors PHOME, E1 and sEH 7 and the bioactivity of their microsomes. Oxidative metabolites were generated by pig liver microsomal incubations in the presence of NADPH according to a modified version of a protocol described elsewhere [26]. In brief, reaction mixtures were prepared in 50 mM potassium phosphate buffer (pH 7.4) including 5 mM magnesium chloride. The mixtures containing 6 mM NADPH, 2.6 mg/mL pig liver microsomes and 100 μM sEH were incubated for 2 h at 37°C. To ensure continued availability of NADPH, 5 mM glucose-6-phosphate and 5 U/mL glucose-6-phosphate dehydrogenase were used as regenerating system. Additionally, 10% (v/v) of a 10 mM NADPH solution in the above mentioned phosphate buffer was added after 30, 60 and 90 min, respectively. The reactions were stopped by adding ice-cold acetonitrile 2:1 (v:v). The samples were then centrifuged at 16,000 g for 5 min. The supernatants were taken, freeze-dried and stored at –20°C. For the LC-BCD/MS analysis, the samples were re-dissolved in a 30% aqueous methanol solution, providing 20-fold higher concentrations.

**Results and Discussion**

**Development of an LC–BCD system for the detection of sEH inhibitors**

The aim of this study was to develop a system for the bioactivity assessment towards sEH of individual compounds in complex mixtures. For this purpose, a homogeneous, continuous-flow detection format was applied based on the enzymatic conversion of the sEH of individual compounds in complex mixtures. For this reason, a modified version of a protocol described elsewhere [26]. In brief, reaction mixtures were prepared in 50 mM potassium phosphate buffer (pH 7.4) including 5 mM magnesium chloride. The mixtures containing 6 mM NADPH, 2.6 mg/mL pig liver microsomes and 100 μM sEH were incubated for 2 h at 37°C. To ensure continued availability of NADPH, 5 mM glucose-6-phosphate and 5 U/mL glucose-6-phosphate dehydrogenase were used as regenerating system. Additionally, 10% (v/v) of a 10 mM NADPH solution in the above mentioned phosphate buffer was added after 30, 60 and 90 min, respectively. The reactions were stopped by adding ice-cold acetonitrile 2:1 (v:v). The samples were then centrifuged at 16,000 g for 5 min. The supernatants were taken, freeze-dried and stored at –20°C. For the LC-BCD/MS analysis, the samples were re-dissolved in a 30% aqueous methanol solution, providing 20-fold higher concentrations.

**Characterization of the LC–BCD system**

The performance of the LC–BCD system to quantitatively measure the potency of sEHs was tested by analysis of five known inhibitors at different concentrations in FIA mode, which is a fast way to measure IC₅₀ values if pure compounds are available. As shown in Figure 4A, the injection of sEH 1 resulted in negative peaks in the BCD chromatogram with increasing negative peak heights upon increasing increasing concentrations. The variance in peak height between duplicate injections was generally lower than 10% (in 90% of 36 samples). As previously shown for various enzymes the negative peak height in LC–BCD systems can be used to calculate the percentage of inhibition [18; 27]. Based on the resulting dose response curves (Figure 4B), it is possible to quantitatively rank the sEHs by their potency. Among the compounds tested, sEH 5 was the most active and sEH 4 the least active inhibitor. In order to deduce IC₅₀ values for each compound, the dilution of the injected amount of inhibitor in the LC–BCD system has to be taken into account [18; 28]. The dilution results from the mixing of LC eluent and BCD reagents (Dₑ) which depends on the flow rates of eluent entering the on-line BCD (uₑ) and the total flow at detection (uₜ).

\[ Dₑ = \frac{uₑ}{uₜ} \]  

Equation 1
In addition, inhibitors injected are also diluted due to their residence time in the flow system ($D_c$). In a test tube or well plate experiment, the inhibitor can be assumed to be evenly distributed after mixing. This phenomenon is a result of longitudinal diffusion of the initially homogeneous injection plug ($V_i$). It further dilutes the injected concentration ($c_i$). The full width at half maximum (FWHM) and the flow rate ($u_f$) have to be derived from the same chromatogram, preferably from the BCD chromatogram which results in $u_c = u_f$.

$$D_c = \frac{FWHM}{2} \cdot \frac{\pi}{\ln(2)} \cdot \frac{u_c}{V_i}$$

Equation 2

Therefore, the final concentration at the maximum negative peak height ($c_p$), which can be calculated from equation 3, was used for the dose-response curves.

$$c_p = \frac{c_i}{D_m \cdot D_c}$$

Equation 3

Thus, the dilution factors are calculated individually for every measurement, and they range from 71 to 210. The final concentrations were plotted against the corresponding percentage of inhibition and the data fitted with GraphPadPrism (GraphPad Software, La Jolla, US). This results in the sigmoidal dose-response curves shown in Figure 4B. The reproducibility of the data points measured (see above) and the quality of the all fits, expressed by resulting $R^2$-values of >0.975, are further indications of the high data quality. The calculated IC$_{50}$ values for the five compounds tested are given in Table 1. They covered a range of about three orders-of-magnitude. This demonstrates that the developed LC–BCD method allows the measurement/detection of highly potent as well as weak sEHIs. Therefore, the final concentration at the maximum negative peak height ($c_F$) and the flow rate ($Vi$ plug ($V_i$)) of eight different concentrations ($c_i$) and six $(D_m)$ were calculated individually for every measurement, and they can be observed with the setup [9]. Most importantly, the newly developed LC–BCD method ranked the potency of the tested inhibitors in the same order as commonly employed end-point assays, except for sEHI 2 (Table 1). However, for few sEHs, the determined IC$_{50}$ differed significantly from literature values: For sEHI 2, the observed potency by LC–BCD was about 20-fold higher compared to the value from an end-point assay, while the measured IC$_{50}$ value for sEHI 4 was about 4-fold higher than previously reported (Table 1). Similar to our observations, up to 20-fold differences have been described between different sEHI assays, because the measured potency for individual sEHs is substrate-dependent [9]. This observation is substantiated with the results for sEHI 1. For this compound, using the same substrate, a very good agreement was found between our data and the literature [7]. The potencies for sEHI 3 and sEHI 5 are also consistent with literature values, despite use of different substrates (Table 1). Overall, the results from the analysis of individual sEHs in the LC–BCD system showed that the negative peak height is a suitable quantitative measure for the potency of inhibitors, and that the data obtained are in good agreement with other methods to characterize the potency of sEHs. In contrast to end-point assays, the LC–BCD system combines identity and activity detection after chromatographic separation. It thus allows assessment of individual compounds in mixtures. This is demonstrated by analysing a mixture of sEHI 6, sEHI 1, sEHI 7, and two compounds without sEHI activity, diclofenac and phenylbutazone. In this case, the BCD signal shows only three major peaks, which corresponded well with the elution times to the three sEHs, whereas in the corresponding LC–UV or LC–MS all five compounds are observed (data not shown). These experiments show that the developed LC–BCD system allows assessing the bioactivity of individual compounds in mixtures. In only one analysis step, it can distinguish between active and non-active compounds in mixtures.

**Application of LC–BCD on the analysis of metabolic mixtures of sEHs**

In order to demonstrate the applicability of the LC–BCD system to the analysis of unknown mixtures, in vitro microsomal incubations of three sEHs were analysed. The LC–MS data showed that each crude extract was metabolized to several metabolites (Figure 5). By combining BCD traces and MS extracted ion chromatograms, the peaks of active compounds can be directly identified and structurally characterized. For sEHI 6, three bioactivity peaks were observed (Figure 5A). The main peak eluting around 43.2 min in LC–MS, peak A4MS, corresponding to peak A4IC in LC–BCD, is

<table>
<thead>
<tr>
<th>Compound</th>
<th>LC–BCD system nM*</th>
<th>Literature values</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>sEHI 1</td>
<td>19 (1)</td>
<td>29 ± 13 nM [7]</td>
<td>PHOME</td>
</tr>
<tr>
<td>sEHI 2</td>
<td>25 (1)</td>
<td>684 nM [22]</td>
<td>CMNPCb</td>
</tr>
<tr>
<td>sEHI 3</td>
<td>12 (2)</td>
<td>15 nM [23]</td>
<td>CMNPCb</td>
</tr>
<tr>
<td>sEHI 4</td>
<td>880 (90)</td>
<td>171 nM [22]</td>
<td>CMNPCb</td>
</tr>
<tr>
<td>sEHI 5</td>
<td>2.6 (0.6)</td>
<td>2 nM [23]</td>
<td>CMNPCb</td>
</tr>
</tbody>
</table>

* Mean and difference (in brackets) from individual fitting of the duplicate curves.

b cyan(2-methoxynaphthalen-6-yl)methyl trans-(3-phenyloxyran-2-yl)methylcarbonate
the parent compound (protonated molecule [M+H]+ with m/z 277.228). The three peaks A1MS, A2MS and A3MS, eluting at 35.0 min, 35.8 min and 38.0 min, respectively, in LC–MS, were not observed in the control incubations (data not shown). These compounds could be tentatively identified as hydroxylated metabolites because all contained an additional oxygen compared to the parent compound ([M+H]+ with m/z 293.223). While peak A1MS is not bioactive, peaks A2MS and A3MS correspond to the peaks AIIBCD and AIIIBCD, respectively. Note that peak A3MS consists of three non-separated compounds thus three different mono-hydroxylated metabolites with m/z 293.209, which were not well separated. The peaks A5MS and A6MS are present as contaminants; they show the same nominal mass as the oxygenated metabolites and the parent sEH 6, but different accurate mass (m/z 293.209 and 277.217, respectively).

The LC–BCD chromatogram of the metabolic incubation trace of sEH 1 showed four peaks (Figure 5B). The main peak BIVBCD corresponds to peak B9BCD, the parent compound ([M+H]+ with m/z 227.214). In this case, six mono-hydroxylated metabolites (B2MS through B7MS, [M+H]+ with m/z 241.208) were observed (Figure 5B). These metabolites gave rise to peaks BIIIBCD and BIIIBCD. By careful evaluation of the peak shapes and retention times, it may be concluded that B2MS and at least two of the metabolites B4MS to B7MS are bioactive. The oxidated dehydrogenated metabolite B1MS ([M+H]+ with m/z 241.192) is clearly not bioactive, whereas the peak shape of the dehydrogenated metabolite B8MS ([M+H]+ with m/z 225.197) matched the retention time of BIIIIBCD. This is an excellent example of the added value of the LC–BCD approach: whereas the minor peak B6MS would be easily ignored in an MS-only approach, its strong corresponding peak BIIIIBCD cannot be overlooked. Compared to sEH 6 and sEH 1, sEH 7 showed less metabolic conversion: only two mono-hydroxylated metabolites were detected ([M+H]+ with m/z 409.306) (Figure 5C). The main peaks C1MS and C3MS correspond to the parent compound ([M+H]+ with m/z 393.312). The two metabolites C1MS and C2MS are not well separated and result in only one peak in the LC–BCD chromatogram. The retention time of C1MS corresponds to the peak C1PCD, indicating the compound is bioactive, but the increased tailing of C1MS suggests that C2 is bioactive as well.

All three sEHs were metabolized in the aliphatic chains and rings at either side of the urea function. A more detailed structural analysis was not possible as MS fragmentation only occurred in or next to the urea function. For all three sEHs tested, LC–BCD/MS analysis allowed the tentative identification of at least two inhibitory active metabolites. In only a single step analysis of 60 minutes, active metabolites can be detected. Moreover, it is possible to distinguish between active and non-active metabolites and to characterize bioactive compounds by their inhibitory potency and MS spectra.

Conclusion

A new LC–BCD system for the detection of sEHs in complex mixtures has been developed. The detection principle is a continuous-flow enzyme activity assay coupled on-line to LC with parallel MS detection. The substrate PHOME allowed sensitive and robust monitoring of bioactivity by fluorescence. After thorough optimization of the assay conditions, the incubation time in the BCD was reduced from 60 to 5.5 min. With a sEH concentration of only 4 nM in the reaction coil, the signal-to-noise ratio for complete sEH inhibition was still higher than 60. Analysis of several known sEHs demonstrated that the peak height, observed in LC–BCD can be used as quantitative measure for sEH inhibition. Moreover, the obtained potencies, measured as IC50 values, for sEHs are in good agreement with previously reported values. The LC–BCD system is able to perform bioactivity analysis of individual compounds in mixtures. This was successfully demonstrated by the analysis of a standard mixture as well as of in vitro metabolic conversions of three known sEHs containing both active and non-active metabolites towards sEH. Here, LC–BCD revealed the formation of new active metabolites, which could be simultaneously characterized by LC–MS.

With the developed LC–BCD system, inhibitors can be detected and characterized in a single analysis. Given the increasing interest in sEH as drug-target for various diseases, this new technique may pave the route for the detection of new classes of sEHs in natural products or crude mixtures arising from organic synthesis. Moreover, metabolism studies with LC–BCD as read out will allow the identification of active metabolites in early stages of lead development and thus assist the identification of the best compounds as drug candidates.

Acknowledgements

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References

Supplementary Material

LC–MS for metabolite identification was done either on a Bruker Daltonik (Bremen, Germany) micrOTOF-Q quadrupole time-of-flight hybrid MS, using the above described conditions, or using an ion-trap time-of-flight mass spectrometer (IT-TOF, Shimadzu, ‘s Hertogenbosch, the Netherlands). In the latter case, a 35-min gradient and a 100 × 2.1 mm Waters XBridge C18 column (3.5 μm particles) was used. In the Bruker quadrupole time-of-flight hybrid MS, an electrospray ionization (ESI) source operated in positive-ion mode at the following settings: capillary voltage 4500 V; end plate offset -500 V; nebulizer gas 1.8 bar; drying gas 8.0 L/min at 200°C. For accurate mass measurements, the TOF settings were automatically calibrated prior to the measurements by infusing a 5 mM sodium formate solution. Mass spectra were collected between m/z 50 and 1000.

In the Shimadzu ion-trap time-of-flight mass spectrometer, the ESI source was operated in positive-ion mode at a needle voltage of 4.5 kV, a temperature of 200°C on the source heating block and the curved desolvation line, a drying gas pressure of 62 kPa and a nebulising gas flow-rate of 1.5 L/min. For accurate mass measurements, the TOF settings were externally calibrated on a weekly basis using sodium TFA clusters. MS spectra were collected between m/z 150 and 650, both with a width of 3 Da and fragmented at 50% CID energy.

The mass accuracy was better than 5 ppm on both instruments. The accurate-mass settings for Shimadzu micrOTOF-Q micrOTOF-Q quadrupole time-of-flight hybrid MS, using the above described conditions, or using an ion-trap time-of-flight mass spectrometer (IT-TOF, Shimadzu, ‘s Hertogenbosch, the Netherlands). In the latter case, a 35-min gradient and a 100 × 2.1 mm Waters XBridge C18 column (3.5 μm particles) was used. In the Bruker quadrupole time-of-flight hybrid MS, an electrospray ionization (ESI) source operated in positive-ion mode at the following settings: capillary voltage 4500 V; end plate offset -500 V; nebulizer gas 1.8 bar; drying gas 8.0 L/min at 200°C. For accurate mass measurements, the TOF settings were automatically calibrated prior to the measurements by infusing a 5 mM sodium formate solution. Mass spectra were collected between m/z 50 and 1000.

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In the Shimadzu ion-trap time-of-flight mass spectrometer, the ESI source was operated in positive-ion mode at a needle voltage of 4.5 kV, a temperature of 200°C on the source heating block and the curved desolvation line, a drying gas pressure of 62 kPa and a nebulising gas flow-rate of 1.5 L/min. For accurate mass measurements, the TOF settings were externally calibrated on a weekly basis using sodium TFA clusters. MS spectra were collected between m/z 150 and 650, both with a width of 3 Da and fragmented at 50% CID energy.

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Section 3
Integration of modification methods and HRS workflows for the study of metabolite-like lead libraries

Chapter 3.1
On-line electrochemistry–bioaffinity screening with parallel HR-LC–MS for the generation and characterization of modified p38α kinase inhibitors

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Abstract

In this study, an integrated approach is developed for the formation, identification and biological characterization of electrochemical conversion products of p38α mitogen-activated protein kinase inhibitors. This work demonstrates the hyphenation of an electrochemical reaction cell with a continuous-flow bioaffinity assay and parallel LC-FC-MS. Competition of the formed products with a tracer (SKF-86002) that shows fluorescence enhancement in the orthosteric binding site of the p38α kinase is the readout for bioaffinity. Parallel HR-MS® purification and LC-ESI-TOF-MS analysis provided information on the identity of binders and non-binders. Finally, the data produced with this on-line system were compared to off-line generated electrochemical conversion products. The electrochemical conversion of 1-(6-chloro-5-((2R,SS)-4-(4-fluorobenzyl)-2,5-dimethylpiperazine-1-carbonyl)-3aH-indol-3-yl)-2-morpholinonethane-1,2-dione (DMPIP) resulted in eight products, three of which showed bioaffinity in the used continuous-flow p38α bioaffinity assay. Electrochemical conversion of BIRB796 resulted, amongst others, in the formation of the reactive quinoneimine structure and its corresponding hydroquinone. Both products were detected in the p38α bioaffinity assay, which indicates binding to the p38α kinase.

Introduction

Electrochemistry (EC) is increasingly described as a tool to support the formation and identification of drug oxidation products [1]. Efforts are reported to mimic a human metabolite profile [2] or to produce specific reactive metabolite species [3]. The application of electrochemical techniques specifically focused at drug development is reviewed by Hillard et al. [4]. These applications are mainly directed at the study of reactive oxygen species, oxidation and reduction of prodrugs, and the alkylation of DNA. Bauman and Karst [5] recently described key principles and use of on-line electrochemical mass spectrometry (EC-MS) in drug metabolism studies. Jurva et al. systematically compared the oxidation of drugs by EC-MS and by cytochrome P450s [6]. This approach provides chemical information on the products formed by electrochemistry and facilitates the comparison with in-vitro incubation models [7, 8]. The chemical information obtained can eventually be used to correlate biological action of already characterized metabolites to the products analyzed. However, this is not feasible for newly formed products for which no biological data are yet available. Still, few examples in EC related drug metabolism studies employ detection of biological activity [9, 10] and, to the best of our knowledge, none tested for a specific target enzyme or receptor. More importantly, in studies employing EC-MS or EC-LC-MS, the information on the products formed by electrochemistry and facilitates the comparison with the efficacy of a drug. For many years, we have been developing hyphenated screening assays to obtain chemical and biological information in a combined manner [11]. This resulted in several approaches to assess bioaffinity, e.g., on-line receptor binding [12], enzyme activity assessment [13], bacterial growth inhibition [14], as well as several other strategies which allowed us to identify and characterize bioactive compounds in complex mixtures. These mixtures included natural extracts, crude synthesis products, medicinal chemistry compound libraries, degradation products by light or harsh chemical conditions as well as in vitro metabolic incubations. The implementation of a device for electrochemical oxidation in our on-line screening platform would lead to a fully automated process of formation of drug-related chemical entities followed by their simultaneous chemical and biological characterization. This leads to a quick feedback between the modifications of a lead compound and their consequences for binding to the drug target. Furthermore, stable and/or reactive products could be analyzed directly after their formation and as such have less chance of degradation.

In this paper, we describe the hyphenation of EC with our recently developed liquid chromatography (LC) - on-line p38α mitogen-activated protein kinase binding assay (p38α bioaffinity assay) with parallel high resolution MS [18]. EC provides relatively clean samples and has shown to facilitate the formation of interesting molecules for drug research [19]. The p38α mitogen-activated protein kinase (p38α kinase) is a prominent example of a drug target kinase [20] and is heavily involved in inflammation processes [21]. The hyphenation of these techniques to develop a fully integrated platform can facilitate the hit-to-lead selection process in drug discovery. This complete hyphenation of EC with LC and ultimately with parallel detection by p38α bioaffinity assay and high resolution MS combines modification with separation, bioaffinity determination and structure elucidation on a new level of integration.

Materials and methods

Chemicals

Acetonitrile, methanol (LC–MS grade) and formic acid (ULC–MS grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Water was produced by a Milli-Q device (Millipore, Alphen aan den Rijn, the Netherlands). Nitrogen 5.0 was purchased from Praxair (Vlaardingen, The Netherlands) and used in all MS experiments. SKF-86002 (SKF) was delivered by Merck KGaA (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) blocking reagent was purchased from Roche Diagnostics (Mannheim, Germany). Ammonium acetate and ammonium hydrogen carbonate were obtained from Mallinkrodt Baker (Deventer, the Netherlands). Fused silica tubing (250-mm inner and 375-mm outer diameter) was covalently coated with polyethylene glycol was obtained from Sigma-Aldrich (Schnelldorf, Germany). Human recombinant p38α kinase, BIRB796, TAK715, 1-(6-chloro-5-((2R,SS)-4-(4-fluorobenzyl)-2,5-dimethylpiperazine-1-carbonyl)-3aH-indol-3-yl)-2-morpholinonethane-1,2-dione (DMPIP) and SB203580 were a kind gift of MSD Research Laboratories (Oss, the Netherlands). Structures of the kinase inhibitor standards used can be found in Figure 1. All other chemicals were from Sigma-Aldrich (Schnelldorf, Germany).

Instrumentation

A schematic representation of the complete on-line setup is shown in Figure 2. The system consists of four modules: (A) an electrochemical reaction cell, (B) an LC system, (C) a continuous-flow bioaffinity assay unit, equipped with a fluorescence detector, and (D) a mass spectrometer. A Roxy electrochemical reaction cell (Antec Leyden, Zoeterwoude, the Netherlands) equipped with a glassy carbon electrode was controlled by the Decade II Potentiostat either manually or under Dialogue software control (Antec Leyden). The 10 mM kinase inhibitor standards, dissolved in 25% ACN and 75% 1 mM aqueous buffer, were infused at a flow rate of 5 mL/min with a Harvard Apparatus (Hollister, USA) syringe pump. The conversion products were collected either in an autosampler vial (‘off-line’ mode) or in 100 mL volume PEEK tubing (‘on-line’ mode) serving as an injection loop mounted in a
remote controlled six port valve (VICI, Schenkon, Switzerland).

In the off-line mode, collected fractions were injected via the autosampler, whereas in the on-line mode the six port valve with the 100 mL PEEEK tubing acted as the injection valve to a Shimadzu LC system (‘s Hertogenbosch, the Netherlands), consisting of two LC-10ADvp pumps, a DGU-14A solvent degasser, a SIL-10A dpv auto injector, two CTO-10ASvp column oven, an SPD-10Avp UV-detector and a SCL-10Avp system controller. The LC separation was performed on a Waters Xbridge C18 column (2.1 mm x 100 mm I.D., 3.5 µm particles). The mobile phases consisted of 99% water with 1% methanol (solvent A) and 1% water with 99% methanol (solvent B), both containing 0.01% formic acid. A generic gradient was applied for the separation: isocratic for 2 min at 90% B until 18 min, isocratic at 90% B until 22 min, a gradient back to 20% B until 23 min and isocratic re-equilibration at 20% B until the end of the run at 30 min. The flow-rate was 113 mL/min. The column temperature was kept at 40°C. All standards eluted with significant retention, which provided sufficient resolution for the separation of products with slightly differing polarities.

Initial off-line experiments were performed with the EC unit and an LC-MS system, without the p38 bioaffinity assay attached, in order to optimize the EC conditions. For this, the LC system was coupled via an electrospray ionization (ESI) interface to a Thermo Finnigan LCQ Deca ion trap mass spectrometer (LRMS) (Breda, the Netherlands). Experimental conditions for the ion trap MS were as follows: Capillary temperature 200°C, sheath gas flow 45 arbitrary units, aux gas flow 5 arbitrary units, source voltage 5 kV and capillary voltage 6 V. The instrument was used in positive-ion ESI mode for full spectra acquisition between m/z 150 and 650.

In on-line experiments with the complete setup, a post-column split was applied (see Figure 2), with 13 mL/min of the LC mobile phase being directed to the p38α bioaffinity assay and 100 mL/min to the MS system. The post-column fluorescence enhancement based bioaffinity assay towards the p38α kinase was identical to the system previously described by us [18]. Important features of the system are shown in Figure 2. Competition of the formed products with a tracer (SKF) that shows fluorescence enhancement in the orthosteric binding site of the p38α kinase is the readout for bioaffinity. Stability of the flow system is essential for the quality of the assay as small changes in the reaction parameters have relatively pronounced consequences in the signal. Therefore, a stable reagent flow is important in order to keep the noise to a minimum. This was achieved by a linear setup of an LC pump, a pulse dampener, a restriction capillary and a superloop. The state-of-the-art LC pump delivered a constant flow of water. The pulse dampener was based on an impermeable membrane and had a comparably extensive water reservoir. The restriction capillary was chosen to produce 1.5 bar of pressure at the applied flow rate of 50 mL/min. As a result, a constant and stable flow entered the superloop served as a reagent reservoir of constant volume which released exactly the same flow rate as it received. Compared to a delivery by the pump alone, reagents were also more evenly cooled and pump maintenance was reduced which further improved the stability of the system.

Parallel to the p38α bioaffinity assay, the electrochemical conversion products were analyzed using a Shimadzu ion-trap time-of-flight hybrid mass spectrometer (LC-IT-TOF-MS), equipped with an ESI source and operated in positive-ion mode to obtain accurate m/z values for [M+H]+ and fragment ions. The ESI needle voltage was set to 4.5 kV, while the source heating block and curved desolvation line temperature were set to 200°C. Drying gas pressure was set at 62 kPa and nebulizing gas was applied at a flow-rate of 1.5 L/min. MS² and MS³ spectra were acquired in data-dependent mode with the following settings: full-spectrum MS with m/z 200 – 650, MS² with m/z 150 – 650 and MS³ acquisition with m/z 100 – 650 with ion accumulation time of 10 ms, precursor isolation width of 3 Da and collision energy set at 75% for TAK715 and 50% for all other compounds. The accurate-mass fragmentation data obtained were used for structure identification. External calibration of the instrument was based on sodium TFA clusters and allowed a mass accuracy lower than 5 ppm. From the measured accurate mass, the elemental composition of the products and their fragment ions were calculated. In order to extract the relevant elemental compositions, restrictions were applied to exclude those which have more atoms of any kind than the substrate plus possible modifications. This is reasonable because the products are linked to the substrate by one or more chemical modifications. For fragments, the elemental composition of the precursor ion was used as restriction. The few compositions left were analyzed for electrochemical modifications possibly leading to the formation of products with a specific composition. Where CID fragments were concerned, this analysis was based on reasonable neutral losses. The procedure was sufficient to restrict the discussion to only one elemental composition.

**Optimization of electrochemical conversion**

For flow-through electrochemical reaction cells, important parameters reported are pH, electrode voltage, organic modifier content and flow-rate [5, 6]. Several techniques can be used to find the optimal conditions. Those include cyclic voltammetry, direct EC-MS coupling, and off-line EC and LC-MS experiments [5, 22]. The latter method was applied in this study. We evaluated per substrate 4 different potentials (0; 0.4; 0.8; 1.2 and 1.5 V) and 4 different pH values (3.5; 5.0; 7.0; 10.0). The four different buffers used to vary the pH in this study. We evaluated per substrate 4 different potentials (0; 0.4; 0.8; 1.2 and 1.5 V) and 4 different pH values (3.5; 5.0; 7.0; 10.0). The four different buffers used to vary the pH were: an ammonium formate / formic acid buffer with pH 3.5, an ammonium acetate / acetic acid buffer with pH 5.0, and two ammonium hydrogen carbonate buffers with pH 7.0 and 10.0, adjusted with formic acid and sodium hydroxide, respectively. Each series resulted in 20 samples. The series was injected in duplicate, which due to the 30 min LC run time resulted in a well defined 10 hrs difference between the two duplicate analyses of the sample. This provided an indication of the stability of the products formed.

From the off-line optimization data, optimum conditions were selected to be applied in the on-line mode, using the complete setup. These conditions are summarized for the three model compounds in Table 1.

**Results and discussion**

In this study, an integrated approach is developed for the formation, identification and biological characterization of conversion products derived from p38α kinase inhibitors. This work demonstrates the hyphenation of an electrochemical reaction cell with a continuous-flow bioaffinity assay and parallel LC-HR-MS. The recently developed p38α bioaffinity...
assay, based on fluorescence enhancement, was used as a model assay to investigate the possibilities of this combination, but the approach is not limited to this specific continuous-flow assay. Parallel HR-MS experiments provide information on the identity of binders and non-binders. Finally, the data produced with this on-line system were compared to that of off-line generated electrochemical conversion products. First of all, the electrochemical conversion of the kinase inhibitors had to be optimized for substrate conversion and product formation. Formation and identification of bioaffinity products is the ultimate goal of the method. High substrate conversion simplifies the bioaffinity assessment by reducing the interference of the unmodified inhibitor. The off-line and on-line approaches employ the same flow-through EC instrument which is operated under the same experimental conditions in both cases. Therefore, the off-line data generated for validation of the on-line setup can be used to extract the optimal electrochemical reaction conditions for both setups. An excerpt of the off-line generated data can be found in the Online Resource figure S2. Although the EC and LC-MS approach can be considered laborious for reaction optimization only, it has advantages over other methods. Cyclic voltammetry will not give any information on product formation and direct EC-MS analysis is very stable (<3% relative standard deviation) [18]. This is expectable with state-of-the-art LC equipment, even more so with reagent delivery through pump, pulse dampener, restriction capillary and superloop which ensures stability of the bioaffinity trace. An increase in peak broadening is due to the sigmoidal dose-response character of the modification sites. Figure 3 shows an overlay of the bioaffinity traces of on-line electrochemistry-LC-p38α kinase inhibition experiments and the relevant extracted ion chromatograms (EIC) from the simultaneous LC-MS experiments for the compound DMPIP (C18H13ClF5N5O4, [M+H]+ with m/z 541.2012). Clearly, bioactive products were formed under all conditions applied, although in different relative concentrations. The four negative peaks in the affinity chromatogram indicate binding/affinity of three conversion products to the p38α kinase by displacing the tracer ligand upon elution from the LC column. The affinity is linked in the same figure to the EIC for identification of the binders. As the products showing affinity are the same under all conditions, the EICs are derived from one measurement. As reported previously, the retention times in the bioaffinity detection as well as in the MS detection are very stable (<3% relative standard deviation) [18]. This is expectable with state-of-the-art LC equipment, even more so with reagent delivery through pump, pulse dampener, restriction capillary and superloop which ensures stability of the bioaffinity trace. An increase in peak broadening is the only difference expected between bioaffinity and MS chromatograms in terms of chromatographic parameters. Even this only applies to highly nonpolar compounds as a result of the low organic modifier content of the bioaffinity assay. Therefore, the bioaffinity and MS chromatograms are matched by their stable retention time difference. The validity of this retention time difference can be double checked if a known binder is present in the mixture, in this case the substrate of the EC. The correlation of a bioaffinity peak with an EIC peak is achieved with high certainty because a margin of twice the maximum relative standard deviation of the retention time, in this case ca 30 s, is applied. If more than two EIC peaks would be matched within ±30 s to a bioaffinity peak, both compounds were possibly responsible for the bioaffinity signal. Fortunately, this case did not occur in the investigation. Please note that bioaffinity chromatograms were correlated with EIC at the front of the peak. In this way, peaks are more easily assessed when overloading of either readout leads to broad peaks with poorly defined maxima. The major affinity peak is due to the unmodified kinase inhibitor, DMPIP. The seemingly large broadening of this affinity peak is due to the sigmoidal dose-response character of the bioassay. Based on the bioaffinity profile, the product with m/z 539.1902 (D – 2.011 u) showed to be the main bioactive product in the oxidative mode. However, in the reductive mode, this product was less abundant and a product with m/z 472.1461 (D – 69.0551 u) also showed significant binding to the p38α kinase. In both the reductive and the oxidative mode, a minor affinity peak was observed corresponding to hydroxylated DMPIP, with m/z 557.1962 (D + 15.9950 u). Identification of the (bioactive) products was done using the HR-MS data obtained in parallel with the bioaffinity measurements. Based on the fragmentation of the unmodified kinase inhibitor, the sites of modification were identified as far as possible. The fragmentation of DMPIP allowed the annotation of characteristic fragments to be used for the determination of the modification sites. Figure 4 shows the structure of DMPIP with the characteristic parts of the molecule involved in the fragmentation reactions. Important fragments observed in the MS/MS spectra of DMPIP are C+D with m/z 319.0480 and C with m/z 206.0003. Table 2 summarizes the identification study. Below, the MS structure elucidation of two ions is discussed in detail. A full description of

<table>
<thead>
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<tr>
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<tr>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>BIRB796</td>
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<tr>
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Figure 3: Correlation of bioaffinity profiles (top) and HR-MS traces (bottom) of electrochemical conversion products related to DMPIP.
The characteristic losses of C and site of the EC modifications drawn from the MS fragmentation is not to be confused with EC modifications (e.g., hydrolysis). For conclusions on nature and site of the EC modifications, please refer to the text. Binding to p38α kinase is defined as an S/N ratio of > 3 in the p38α bioaffinity assay (see also figure 3).

<table>
<thead>
<tr>
<th>T in min.</th>
<th>[M+H]+</th>
<th>Change in formula</th>
<th>Molecular formula</th>
<th>MSn</th>
<th>AB</th>
<th>C</th>
<th>CD</th>
<th>other fragments</th>
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<tr>
<td>16.1</td>
<td>541.2012</td>
<td>-</td>
<td>C28H28N4O21</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9.3</td>
<td>431.1481</td>
<td>-C,H,F</td>
<td>C24H24N2O2</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>11.8</td>
<td>501.1699</td>
<td>-C,H</td>
<td>C29H26N3O2</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>BC-CH4</td>
</tr>
<tr>
<td>12.8</td>
<td>525.1699</td>
<td>-CH2</td>
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<td>-</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14.0</td>
<td>472.1461</td>
<td>-CH2</td>
<td>C29H26N3O2</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>15.6</td>
<td>557.1962</td>
<td>+O</td>
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<td>+</td>
<td>O</td>
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<td>529.1649</td>
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<tr>
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<td>-2H</td>
<td>C29H27N3O2</td>
<td>-</td>
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<td>√</td>
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MS fragmentation is not to be confused with EC modifications (e.g., hydrolysis). For conclusions on nature and site of the EC modifications drawn from the MS data, please refer to the text. Binding to p38α kinase is defined as an S/N ratio of > 3 in the p38α bioaffinity assay (see also figure 3).

The products showing bioaffinity are underlined in gray.

The structure elucidation for all ions observed is avoided in order to focus the discussion on the innovative combination of techniques. As an example, the MS and MS/MS spectra for the bioactive product with m/z 472.1461 are given in figure 5. The m/z difference compared to the parent compound is D = -69.0551 u, consistent with a net loss of CH2N. The data indicate that the molecule is modified in the D part, see figure 4. Parts A+B and C are detected unmodified, whilst the fragment of C+D shows the net loss of CH2N. Fragmentation of the [M+H]+ additionally results in the neutral loss of CO2, leading to an unmodified B+C fragment. The characteristic losses of CH2N and CO2 prove the hydrolysis of the amide in part D to a carboxylic acid, which is further supported by the MS2 fragments (data not shown). Therefore, full structure elucidation could be achieved by MSn analysis for this product. For the product with m/z 539.1902, attributed to a dehydrogenation of part B, absolute identification is not possible. The attribution is made by exclusion. As fragment CD is observed unchanged, modification in parts C and D is excluded. Since the dehydrogenation is observed in a BC fragment and C has already been excluded, the structure of DMPiP and characteristic parts for MS/MS identification of electrochemical conversion products.

Table 2: Identification of electrochemical conversion products related to DMPiP based on high resolution MSn experiments (for explanation: see text). MS fragmentation is not to be confused with EC modifications (e.g., hydrolysis). For conclusions on nature and site of the EC modifications drawn from the MS data, please refer to the text. Binding to p38α kinase is defined as an S/N ratio of > 3 in the p38α bioaffinity assay (see also figure 3).

The bioaffinity assessment of the products based on the inhibitor BIRB796 (C31H28N2O3, [M+H]+ with m/z 472.1461) and m/z 557.1962) as well as dehydrogenation in part B (m/z 539.1902) of DMPiP all retained the affinity for the p38α kinase, see table 2. Products not showing bioaffinity include methoxylation of part A (m/z 571.2110, C29H26N3O2; see also below), cleavage of methyne from part B (m/z 525.1720, C29H26N3O2) and the loss of C,H from part B (m/z 501.1715, C29H26N3O2). The electrochemically induced cleavage of part A resulted in a product with m/z 431.1476 (C29H24N3O2). The bioaffinity assessment of the products based on the inhibitor BIRB796 (C31H28N2O3, [M+H]+ with m/z 528.2975) showed that next to BIRB796 itself, two bioactive electrochemical conversion products were formed. Figure 6 shows the extracted ion chromatograms of the three bioactive compounds aligned with the bioaffinity trace. The two additional bioactive compounds were the main conversion products, being identified as the quinoneimine (m/z 413.1985, C29H26N3O2; D 115.0990 u) and the hydroquinone (m/z 415.2134, C29H26N3O2; D 113.0841 u), both being present in all conversion samples. Interestingly, Regan et al. described the structure-activity relationships of BIRB796 in two of its fragments [23]. The hydroquinone was found to bind to the kinase, while the reactive quinoneimine was addressed neither in this publication nor elsewhere in literature. The fact that we can detect bioaffinity of the reactive quinoneimine underlines the importance of a quick and clean sample handling technology between formation and analysis of the conversion products. In this respect, our on-line approach can be of added value with respect to the assessment of the affinity of reactive species. Other electrochemical conversion products of BIRB796 were not bioactive. These included a product with m/z 230.1652.

Figure 4: Structure of DMPiP and characteristic parts for MS/MS identification of electrochemical conversion products.

Figure 5: MS and MS/MS spectra of the electrochemical conversion product of DMPiP, with m/z 472.1461. Annotations related to the parent structure (figure 4) are included in the spectra.
corresponding to \( \text{C}_9\text{H}_{17}\text{N}_4\text{O}_{5} \) and a low abundant product with \( m/z \) 273.1710 corresponding to \( \text{C}_9\text{H}_{16}\text{N}_4\text{O}_{5} \) eluting with the unmodified BIRB796. Structure proposals can be found in the Online Resource figure S1. Oxidation at pH 7.0 and 10.0 resulted in two additional products with \( m/z \) 386.1883 (\( \text{C}_9\text{H}_{16}\text{N}_4\text{O}_{5} \)) and 411.1821 (\( \text{C}_9\text{H}_{17}\text{N}_4\text{O}_{5} \)).

For SB203580 and its conversion products, affinity determination was complicated by the auto-fluorescence of SB203580 and its products at the wavelengths used for the detection of the enzyme-tracer complex in the p38α bioaffinity assay. Electrochemical conversion of SB203580 (\( \text{C}_9\text{H}_{17}\text{FN}_3\text{O}_5^+ \) with \( m/z \) 378.1071) resulted in the formation of single (\( m/z \) 394.1026) and double oxygenated analogues (\( m/z \) 410.0969). The main product could be identified as the sulfone product based on its fragmentation pattern described previously by Henklova et al [24]. The rather scarce fragmentation of SB203580 did not allow proposing structures of the other products generated by electrochemical conversion. In order to compare the off-line conversion with the conventional off-line generated products, a set of off-line electrochemical conversion experiments was conducted. This included variation of pH and electrochemical potential for the conversion of the inhibitors. The samples were measured twice, once immediately after generation and once with an additional storage time of 10 hours. In this way, a focus was set on the effects of storage as a major difference between the off-line and on-line approach. For DMPIP and SB203580, no differences were observed in the conversion profiles between off-line and on-line conversion and analysis. This means that product formation is comparable between the two modes. The results of the off-line experiments also help to explain the origin of the methoxylated product for DMPIP. It may be suggested that the methoxylation does not take place in the EC cell, but rather via a reaction of a reactive intermediate with methanol in the LC mobile phase. However, the off-line experiments provide evidence against this. The off-line data, shown in figure S3, are not consistent with an intermediate reaction with the LC eluent, because, if it would be reacting, the amount of methoxylated product should be the same or less after storage (depending on the stability of the intermediate). However, the data actually show a higher abundance of methoxylated product after storage which indicates a reaction of an intermediate within the EC reaction mixture. Furthermore, if a reaction would take place during the chromatographic step, the peak shape of the methoxylated product would be seriously deteriorated. This is not observed. For BIRB796, differences were observed between off-line and on-line conversion products. The peak area of the reactive quinoneimine significantly decreased in the duplicate measurement after 10 hrs (see Online Resource figure S2), whilst in the pH 7 samples the hydroquinone was only present after 10 hrs. The products with \( m/z \) 230 and \( m/z \) 273 had an increased peak area in all duplicate samples and can therefore be either considered as secondary conversion products or degradation products. This is a direct consequence of the storage and handling steps and can be avoided in the on-line approach presented in this paper.

## Conclusion

Electrochemical conversion of drug molecules has already been demonstrated to be a valuable tool to assist in drug metabolite identification studies. As an additional valuable feature, we showed that electrochemistry coupled on-line with LC-MS/bioassay can be used for the generation of small focused libraries of bioactive structures closely resembling the parent structures and assessment of their bioaffinity profile. The hit rate in these focused libraries can be significant. With the integration of direct affinity assessment of the electrochemical conversion products, one can quickly determine critical positions of a molecule influencing its affinity towards the target protein. Moreover, the on-line hyphenation of formation with identification and affinity determination strategies allows the affinity assessment of reactive species since sample handling steps and times are significantly reduced. The latter was clearly demonstrated by the affinity determination of the reactive quinoneimine of the BIRB796 inhibitor. All of these results stress the importance of employing biological characterization as an integral part of EC studies concerned with drug metabolism or lead discovery/optimization.

In principle, the on-line integrated setup could be expanded to an automated system applicable in routine analysis. Instead of by infusion, the parent compounds could be flow-injected into the electrochemical reaction cell. Solvent select valves could be used for convenient and automated changing of the solvent composition and pH. Evidently, the subsequent electrochemical conversion can also be fully automated and controlled via the appropriate controlling software during the analytical runs. The complete setup should be capable of implementation in fully automated and integrated analytical workflows in drug discovery laboratories.

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23. Supplementary Material

Figure S1: Structure proposals for the BIRB796 related conversion products. The main products are the hydroquinone and the quinoneimine. Both showed bioaffinity. The bioaffinity detection of the reactive quinoneimine species was especially noteworthy. Furthermore, two deamination products with m/z 273.1710 and 230.1652 were observed which did not show bioaffinity. In the case of BIRB796, the MS structure elucidation of the breakdown products is very precise. The elemental compositions explain only the modification to one specific isomer in contrast to for example the hydroxylpyridone product of DMP1P where multiple isomers are possible.
Figure S2: Product profiles of BIRB796 for the off-line conversion. The x-axis gives the varying EC reaction conditions being different pH and voltage. For every pH/voltage pair and every ion, there are two rows in the x-dimension, the left one representing the first measurement and the right one the measurement with an additional storage time of 10h. The y-axis gives the area of MS response in arbitrary units calculated from the total ion current (A) or the extracted ion currents (B and C).

A) The area of BIRB796 is calculated from the total ion current because of the coexistence of the single- and double-charge protonated molecule. This indicated stability of the MS response over time as well as the chemical stability of BIRB796.

B) The highest signals at low pH are obtained from m/z 413.1 (black and dark blue) and 415.2 (grey and light blue) later identified as the quinoneimine and the hydroquinone, respectively. Their signals are lower at every pH in the x+10h sample (dark and light blue) than in the immediate measurements (black and grey). This hints to instability or reactivity which is in line with the structures proposed.

C) The opposite effect is observed for the analytes with m/z 273.2 (black and dark blue) and 230.2 (grey and light blue). Their response is always higher after additional storage (dark and light blue) which implies their formation by a secondary reaction or degradation step. The apparent changes in the profile under off-line conditions emphasize the need to avoid liquid handling and storage steps.

Figure S3: Product profiles of DMPIP for the off-line conversion. The x-axis gives the varying EC reaction conditions being different pH and voltage. For every pH/voltage pair and every ion, there are two rows in the x-dimension, the left one representing the first measurement (black) and the right one the measurement with an additional storage time of 10h (blue). The y-axis gives the area of MS response in arbitrary units calculated from the extracted ion currents.

A) The area calculated from the MS response of DMPIP is stable over time. Only the samples at pH 3.5 at 0 and 800 mV show a large deviation. As figure B and C show no response at these conditions, this is not relevant for the analysis.

B) The product with m/z 539.1 which showed the highest affinity in the later on-line analysis is presented here. It was identified as the product of a dehydrogenation in the dimethylpiperazine ring of DMPIP. It seems to be stable at high pH, but instable at low pH.

C) The product of methoxylation (m/z 571.0) has a higher response after storage. As explained in the Discussion, this indicates the reaction of an intermediate with the EC mixture and excludes interference of the eluent. It also seems to be pH dependent as the response is more similar at higher pH.
Chapter 3.2

Combination of biotransformation by P450 BM3 mutants with on-line post-column bioaffinity and mass spectrometric profiling as a novel strategy to diversify and characterize p38α kinase inhibitors

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Abstract

Mutants of bacterial Cytochrome P450 BM3 from Bacillus megaterium have gained increasing interest to support drug metabolism studies by producing libraries of new chemical entities based on initial lead compounds. In this study, a library of 33 P450 BM3 mutants was used to diversify TAK-715, a representative (highly lipophilic) inhibitor of p38α mitogen-activated protein kinase (p38α). To simultaneously determine the individual bioaffinity and identity of the different products, an analytical high-resolution screening approach was used, based on post-column on-line bioaffinity profiling with parallel mass spectrometric identification. The screening of the product mixtures produced demonstrated that introducing mutations in the active site of the P450 BM3 resulted in different product profiles. Several P450 BM3 mutants were mimicking the metabolic profile obtained by human liver microsomes. The major biotransformation products of TAK-715 could be produced with the most active P450 BM3 mutant in sufficient amounts to enable further structure elucidation by 1H-NMR and quantification of their p38α affinity.

Introduction

A relatively new development in drug discovery is the application of biocatalysts for the generation of new chemical entities or even unique compound libraries [1,2]. When using monoxygenase systems, such as the cytochrome P450 enzymes (CYPs), the biotransformation products are particularly interesting in lead optimization as they can show improved physicochemical properties, such as solubility, and target selectivity without significant loss of their pharmacological activity compared to the parent itself. CYPs are also involved in the majority of drug metabolism reactions: in particular CYP1A2, CYP2C9, CYP2C19 and CYP3A4 are responsible for the bulk of metabolic activation of known drugs in humans [3]. Because of the potential pharmacological activity of drug metabolites, safety studies on circulating metabolites are required [4]. As a consequence, organic synthesis is needed to obtain sufficient amounts of pure compound. Biosynthetic approaches are upcoming alternatives to organic synthesis. Biocatalysts, i.e., enzymes, can perform reactions with a high degree of regio- and stereo- selectivity that often cannot be achieved by organic synthesis [5,6]. Obviously, CYPs are emerging as biocatalysts due to their catalytic versatility and extremely wide substrate diversity [7,8]. Here, bacterial CYPs are preferred to mammalian CYPs because they are soluble, show much higher turnover rates and can be expressed at high levels in E. coli. A very promising candidate as biocatalyst for the generation of large quantities of biotransformation products is the drug is P450 BM3 (CYP102A1) from Bacillus megaterium. This enzyme shows the highest catalytic activity ever recorded for a CYP [9]. Recently, many research groups have succeeded in expanding the substrate selectivity of P450 BM3 and improving its catalytic properties by site-directed and/or random mutagenesis, as recently reviewed [10]. Although several P450 BM3 mutants can perform oxygenation reaction with a high degree of regio- and stereoselectivity [11,12], biosynthesis of drug metabolites using engineered P450 BM3s can lead to the formation of multiple metabolites [1,13-15]. Structural identification and characterization of pharmacological and toxicological properties of the individual components in such mixtures requires elaborate semi-preparative fractionation. Alternatively, characterization of compounds in complex mixtures can be performed using the high-resolution screening (HRS) approach, that combines analytical separation with post-column on-line bioaffinity profiling, based on enzyme inhibition or displacement of tracer compounds, and parallel identity assessment with mass spectrometry (MS). This approach has been successfully used for the identification of ligands/inhibitors of several drug targets, such as human estrogen receptors α and β, acetylcholinesterase, acetylcholine binding protein, proteases and peptidases, and drug metabolizing enzymes such as CYPs and glutathione S-transferases [16]. The combination of P450 BM3-mutants as biocatalyst and a HRS platform has been previously used to study activity of metabolites of norethisterone and the mycotoxin zearalenone to the ligand binding domains of (human) estrogen receptors (hER) as drug targets [17,18]. Recently, a HRS methodology has been developed for the on-line screening of inhibitors of p38α mitogen-activated protein kinase (p38α) with simultaneous structure elucidation by high-resolution MS (HR-MS) [19]. The p38α is an important drug target in contemporary drug discovery because it plays a central role in inflammatory cellular signalling processes [20]. Several companies have reported preliminary human clinical results for p38α inhibitors, but until now no drug has reached the market [21]. In the present study, a library of P450 BM3 mutants was used to prepare mixtures of biotransformation products of the p38α inhibitor TAK-715 (N-[4-[2-ethyl-4-(3-methylphenyl)-1,3-thiazol-5-yl]-2-pyridyl]benzamide, see Figure 3) and to characterize these using the p38α HRS platform. The high turnover rates of P450 BM3s are an advantage in the production and purification protocols for these enzymes allow semi-preparative production of human relevant metabolites or new chemical entities for further pharmacological profiling [1]. TAK-715 was one of the most potent p38α inhibitors from a series of synthetic 4-phenyl-5-pyridyl-1,3-thiazoles [22,23]. TAK-715 was advanced to clinical Phase II trials for rheumatoid arthritis, but was discontinued [24]. Recently Verkaar et al. showed that TAK-715 is able to inhibit the Wnt-3a-stimulated β-catenin signalling pathway [25] whose aberrations are associated with several malignancies, most notably cancer [26]. Therefore, lead diversification of TAK-715 is still relevant. Previously reported lead optimization of typical p38α inhibitors was performed using a library encompassing rather lipophilic compounds. Application of the P450 BM3 mutant mono-oxygenases is anticipated to improve the physicochemical properties of the lead compounds, while maintaining their bioaffinity.

Materials and methods

Chemicals

Human recombinant p38α and TAK-715 were a kind gift of MSD Research Laboratories (Oss, the Netherlands). SKF86002 was delivered by Merck KGaA (Darmstadt, Germany). Fused silica tubing (250-μm inner and 375-μm outer diameter) covalently coated with polyethylene glycol (PEG) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Methanol (LC–MS grade) and formic acid (LC–MS grade) were from Biosolve ( Valkenswaard, the Netherlands). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Schnelldorf, Germany). Human liver microsomes (HLM) pooled from different individual donors were obtained from BD Gentest TM (San Jose, USA) and contained 20 mg/mL protein (Cat. No. 452161).

Expression and isolation of P450 BM3 mutants

Expression of the CYP 102A1 mutants was performed by transforming competent E.Coli BL21 cells with the corresponding pET28+–vectors, as described previously [27]. For expression, 600 mL Terrific Broth (TB) medium (24 g/L yeast extract, 12 g/L tryptone, 4 mL/L glycerol) with 30 mg/mL kanamycin was inoculated with 15 mL of an overnight culture. The cells were grown at 175 rpm and 37°C until the OD600 reached 0.6. Then, protein expression was induced by the addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The temperature was lowered to 20°C and 0.5 mM of the heme precursor 5-aminolevulinic acid was added. Expression was allowed to proceed overnight. Afterwards, cells were harvested by centrifugation (4600 × g, 4°C, 25 min), and the pellet was resuspended in 20 mL Kpi-glycerol buffer (100 mM potassium phosphate (KPi) pH=7.4, 10% glycerol, 0.5 mM EDTA, and 0.25 mM dithiothreitol). Cells were disrupted by French press (1000 psi, 3 repeats) and the cytosolic fraction was separated from the membrane fraction by ultracentrifugation of the lysate (120,000 × g, 4°C, 60 min). CYP concentrations were determined using a carbon
monoxide (CO) difference spectrum assay.

**Selection of the P450 BM3 mutant library**

In the present study, 33 mutants of P450 BM3 were selected which could be expressed at good levels and which showed catalytic diversity towards a variety of drugs and steroids. Mutants M01 (R47L, F87V, L188Q, E267V, G415S), M02 (R47L, L86I, F87V, L188Q, N319T), M05 (R47L, F81I, F87V, L188Q, E267V, G415S) and M11 (R47L, E64G, F81I, F87V, E143G, L188Q, E267V, G415S) were previously constructed by a combination of three site-directed mutations and subsequent random-mutagenesis by error-prone PCR [13]. Mutants M01 and M11 were used as templates for additional site-directed mutagenesis of active site residues, guided by available crystal structures of P450 BM3. In our previous studies, we mutated this position in M11 to all possible amino acids, showing that mutation at position 87 has been extensively studied as the amino acid lies very close to the heme, according to available crystal structures of P450 BM3. In our previous studies, we selected for mutagenesis were residues 72, 74, 82, 87 and 437 which appear to be key active site residues which have profound influence on regio- and stereoselectivity of P450 BM3 mutants. Position S72 and A74 are both located in the substrate binding channel [7,29] and have been shown to influence regioselectivity and activity [15,30]. The effect of a negatively charged residue (Asp or Glu) in both M01 and M11 in position 72 and 74 is evaluated in this work.

Mutation A82W was selected because it strongly influenced regioselectivity of steroid hydroxylation when applied to M01 and M11 [11].

Mutations at position 87 have been extensively studied as the amino acid lies very close to the heme, according to available crystal structures of P450 BM3. In our previous studies, we mutated this position in M11 to all possible amino acids, showing that mutation at position 87 has been extensively studied as the amino acid lies very close to the heme, according to available crystal structures of P450 BM3. The position was selected for mutagenesis because residues 72, 74, 82, 87 and 437 which appear to be key active site residues which have profound influence on regio- and stereoselectivity of P450 BM3 mutants. Position S72 and A74 are both located in the substrate binding channel [7,29] and have been shown to influence regioselectivity and activity [15,30]. The effect of a negatively charged residue (Asp or Glu) in both M01 and M11 in position 72 and 74 is evaluated in this work.

Biotransformation of TAK-715 by P450 BM3 mutants and human liver microsomes

Incubations of TAK-715 were performed in 100 mM Pi buffer pH 7.4 with 250 nM P450 BM3 mutants. The final volume of the incubation was 200 µL with 100 µM substrate concentration. The reactions were initiated by addition of a NADPH regenerating system with final concentrations of 0.2 mM NADPH, 20 mM glucose-6-phosphate and 0.4 U/mL glucose-6-phosphate dehydrogenase. The reaction was allowed to proceed for 60 min at 25°C and was terminated by the addition of 200 µL of cold methanol. For human liver microsomes (HLM), a final microsomal protein concentration of 5 mg/mL was used and the incubations were performed as described above, at 37°C instead of 25°C. Precipitated protein was removed by centrifugation (15 min, 14000 × g), and the supernatant was analyzed by UPLC using an Agilent Technologies 2000 system and a Zorbax Eclipse XDB-C18 column (1.8 mm, 50 mm × 4.6 mm; Agilent Technologies, USA). The gradient used was constructed by mixing the following mobile phases: eluent A (0.8 % methanol, 95 % water, and 0.2 % formic acid); eluent B (99 % methanol, 0.8 % water, and 0.2 % formic acid) with a flow rate of 1 mL/min. The gradient was programmed as follows: from 0 to 8 minutes linear increase of eluent B from 40% to 100%; from 8 to 9 minutes, isocratic 100% B; from 9 to 9.5 minutes linear decrease from 100% to 40% of eluent B; from 9 to 12 minutes isocratic 40% eluent B. The products and substrate were detected at 254 nm.

**HRS analysis of biotransformation products of TAK-715 using the LC-p38a kinase affinity/MS platform**

The HRS analysis of the biotransformation products is conducted with an LC–enzyme binding detection/MS platform which has been described previously [19]. The platform comprised a LC–MS system from Shimadzu (’s Hertogenbosch, the Netherlands), including two LC-20AD and two LC-10AD isocratic pumps, an SIL-20AC, a CTO-20AC and a CTO-10AC column oven, an RF-10AXL fluorescence detector, an SPD-AD UV/VIS detector, and a CBM-20A controller coupled to an ion-trap time-of-flight hybrid mass spectrometer for HR-MS equipped with an electrospray ionization (ESI) source. In short, the biotransformation product mixtures were separated on an Xbridge C18 column 100×2.1 mm with 3.5 µm particles (Waters, Milford, MA, USA) at 40°C and a flow rate of 113 µL/min. The gradient used was constructed by mixing the following mobile phases: eluent C (1 % methanol, 99 % water, and 0.01 % formic acid); eluent D (99 % methanol, 1 % water, and 0.01 % formic acid). The gradient was programmed as follows: from 0 to 2 minutes isocratic at 20 % D; from 2 to 18 minutes, linear increase of eluent D from 20% to 90%; from 18 to 22 minutes, isocratic 90% D; from 22 to 23 minutes linear decrease from 90% to 20% of eluent D; from 23 to 23 minutes re-equilibration isocratic at 20% eluent D. Post-column, the flow was split in a ratio of 1:9 directing 13 µL/min to the bioaffinity detection and 100 µL/min to UV/VIS and ESI-HR-MS analysis. The bioaffinity detection is based on competition of analytes with a tracer showing fluorescence enhancement. Affinity towards p38a is assessed by mixing the analytes with 45 mM of enzyme and 630 nM of tracer, SKF86002. The readout occurred at excitation 355 nm and emission 425 nm, 15 nm bandwidth each, in the flow-through fluorescence detector. The UV/VIS detector was operated in dual wavelength mode at 210 nm and 254 nm. For ESI, the needle voltage was set to 4.5 kV and the source heating block and the curved desolvation line were kept at 200°C. A drying gas pressure of 62 kPa and a nebulizing gas flow-rate of 1.5 L/min assisted the ionization. Full spectra were obtained in the positive-ion mode between m/z 200 and 650. MS¹ and MS² spectra were obtained in data-dependent mode between m/z 100 and 650 with an ion activation of 0.1 eV at a precursor isolation width of 25%. The IC₅₀ determinations were done in flow-injection analysis (FIA) mode, which means the separating column was replaced by a low dead volume union (VICI, Schenkon, Switzerland). The volume time peak reduces analysis time and minimizes conversion to a column elution without retention. In these experiments, an injection volume of 50 µL was used to achieve adequate final concentrations for full inhibition. Negative peak heights were plotted against the corresponding final concentrations. The latter were calculated as described earlier [19].

**Large-scale production and isolation of biotransformation products by preparative HPLC**

The biotransformation products of TAK-715 were produced on a semi-preparative scale by incubation with the most active P450 BM3 mutant as biocatalyst. A 100 mL reaction volume containing 1 µM P450 BM3, 100 µM TAK-715 and NADPH regenerating system (as described above) was prepared in 100 mM PiK Pi buffer at pH 7.4. The reaction was allowed to continue for 5 h at 25°C. To achieve maximal conversion of TAK-715, the incubation was supplemented every hour with 3 mL of 30 µM P450 BM3 M11 and 5 mL NADPH regenerating system (20× concentrated). The reaction mixture was extracted three times by 100 mL ethyl acetate. The combined organic layers were collected in a round-bottom flask and evaporated to dryness using a rotary evaporator. The residue was redissolved in 10 mL of 50% MeOH/H₂O and applied by manual injection on a preparative chromatography column Luna 5 µm C18 (250 × 100 mm i.d.) (Phenomenex, Torrance, CA, USA), which was previously equilibrated with 40% eluent B. A flow rate of 2 mL/min and a gradient using the eluents A and B was applied for separation of transformed products. The gradient was programmed as follows: from 0 to 40 min linear increase of eluent B from 40% to 100%; from 40 to 50 min isocratic 100% B, from 50 to 55 min linear decrease to 40% B, and then re-equilibration was maintained until 65 min. Biotransformation products were detected using UV detection at 254 nm and collected manually. Collected fractions were...
first analyzed for purity and identity by the analytical UPLC. Fractions containing individual metabolites were evaporated to dryness under nitrogen stream and dissolved in 1 mL deuterium oxide to exchange acidic hydrogen atoms by deuterium atoms. Finally, after drying by a SpeedVac evaporator, the residues were redisolved in 500 µL of methanol-d₄ and ¹H-NMR spectra were recorded at room temperature. ¹H-NMR-analysis was performed on Bruker Avance 500 (Fallanden, Switzerland) at 500.23 MHz. Afterwards, the samples were dried and redissolved in 30% MeOH and subjected to LC–enzyme binding detection/MS analysis. LogP values were calculated with ChemBioDraw Ultra version 12 from the structures obtained.

Results and discussion

Biocatalysed synthesis of lead compounds from TAK-715 by P450 BM3 mutants; comparison to human liver metabolism

TAK-715 was incubated for 60 min with a library of 33 mutants of bacterial P450 BM3 and pooled human liver microsomes (HLM), and subsequently analysed by UPLC. The results of these incubations, including the percentage of conversion of substrate and profile of metabolites, are tabulated in Table S1 of the ESI. As shown in Figure 1, in total eight metabolites were found after incubation of TAK-715 with HLM, encoded TAK-P1 to TAK-P8 based on their order of elution, whereas six products were observed with P450 BM3 (TAK-P3 and TAK-P4 were not observed). The P450 BM3 only produced human relevant metabolites of TAK-715. The most active P450 BM3 were M11 and its mutants at position 74 and 437. M11 produced TAK-P7 as major product, representing 47 ± 3% of the total metabolism, whereas TAK-P1, TAK-P2, TAK-P6 and TAK-P8 were formed at 13% or less. Mutation to a negatively charged amino acid (Asp or Glu) or hydrogen bond donor (Thr) in positions 74 or 437 of M11 has little to no effect on activity but changed the profile significantly by producing threefold higher amounts of TAK-P1, making this the major product (Table S1).

In contrast, mutant M11 V87I did not produce any TAK-P1, making it more selective to produce TAK-P7 (60% of total biotransformation). TAK-P5 turned out to be the major product for 22 out of the 33 P450 BM3-mutants studied (Table S1). In particular, the mutants showing relatively low activity appeared to be highly selective in the production of TAK-P5. The only mutant which showed a significantly different profile was M11 L437N which was the only mutant to produce TAK-P6 as the major product (38%). Based on its high activity and its ability to produce most of the products at significant amounts, M11 was selected to further evaluate the affinity of the TAK-715 biotransformation products towards p38α in the on-line HRS-affinity assay and to perform a large-scale incubation to obtain sufficient amounts of the products for ¹H-NMR and further affinity assessments with individual compounds.

From Table S1, it can also be concluded that the P450 BM3 mutants were able to form almost all human-relevant metabolites although with different activities and with strongly different product ratios. After 60 minutes of incubation, 33 ± 2 % of the substrate was converted by HLM, corresponding to 25.2 nmol product/min/CYP. The main metabolite was TAK-P7, which represented 29 ± 3% of the total metabolism, respectively. Metabolites TAK-P1, TAK-P2, TAK-P5 and TAK-P6 were formed at comparable amounts by HLM, ranging from 14 to 20% of the metabolites, whereas the minor metabolites TAK-P3, TAK-P4 and TAK-P8 were formed at 3 to 5% of total metabolism.

Parallel on-line bioaffinity assay and mass spectrometry of TAK-715 biotransformation products produced by P450 BM3 M11 and HLM

Although HLM incubations are not in the focus of this investigation, they produce the same products as BM3 M11 as well as a few other minor products. Affinity analysis was performed for both HLM and P450 BM3 M11 incubations. Figure 2A shows the extracted-ion and bioaffinity chromatograms for the TAK-715 metabolites produced by HLM, whereas Figure 3B shows the same for the products generated by BM3 M11. Retention time differ from the chromatograms shown in Figure 1, because another HPLC system was used. However, the order of elution was unchanged. Next to TAK-715, four of its biotransformation products and eight of its metabolites showed affinity towards p38α (Figure 2). Data on these compounds are summarized in Table S1. As expected, TAK-715 (eluting at 38.5 min, [M+H]+ with m/z 400.151) showed a strong bioaffinity. The second strongest decrease in fluorescence was found at 33 min, closely corresponding to TAK-P6 (32.7 min) and TAK-P7 (33.0 min). The lower resolution of the HRS-system did not allow to attribute the bioaffinity signal to any of these metabolites. MS analysis showed that TAK-P6 and TAK-P7 with m/z 416.146 and 430.123 correspond to a mono-oxygenated TAK-715 and double oxygenated/dehydrogenated TAK-715, respectively. The mono-oxygenated product TAK-P5 (31.4 min, m/z 416.146) and the double oxygenated products TAK-P1 (23.0 min, m/z 432.138) and TAK-P4 (27.8 min) also showed bioaffinity. TAK-P3 (24.3 min, m/z 430.123) only showed a very weak bioaffinity signal. The products TAK-P2 (24.3 min, m/z 446.121) and TAK-P8 (34.5 min, m/z 416.146) did not show significant bioaffinity.

Next to the eight metabolites previously found by UPLC, two additional metabolites were identified based on small bioaffinity peaks in the HRS system. TAK-P9 (8.4 min, m/z 312.119) can be rationalized by hydrolysis of the amide-bond of one of the mono-oxygenated TAK-715 products. TAK-P10 (17.4 min, m/z 448.138) apparently results from triple oxidation of TAK-715. The bioaffinity signal at 4.5 min appeared to be unrelated to TAK-715.

Preparative scale biosynthesis of TAK-715 biotransformation products

To obtain sufficient amounts of TAK-715 biotransformation products for NMR structural identification and affinity testing of isolated products to p38α, a large-scale (100 mL) incubation was performed of TAK-715 with mutant P450 M11. Figure S1 of the ESI shows the preparative HPLC chromatogram of the product mixture obtained. After 5 hours of incubation, with hourly additions of enzyme and cofactors, almost 90% conversion of 100 µM of TAK-715 was obtained. LC–MS/MS measurements were performed to confirm the
identity of isolated products. The main product TAK-P1 (35.7 min) represents 39% of the total biotransformation and corresponds to 1.5 mg of product formed. Two mono-oxygenated products (TAK-P5 and TAK-P6, 42.6 min and 43.5 min) represent 27% (1.0 mg) and 12% (0.4 mg) of the total biotransformation, respectively. TAK-P7 (43.8 min) represents 15% of the total biotransformation (0.6 mg), whereas TAK-P2 (37 min) was formed at less than 5%. Interestingly, also a small amount of TAK-P4 was found in the large-scale incubation. The relative product amounts obtained by large-scale incubation are slightly different from that obtained in the screening experiment because of the longer incubation time (5h vs. 1h) and higher enzyme/cofactor concentration. Also, the less efficient aeration of large-volume incubations might contribute to the different profile.

Structure elucidation of TAK-715 biotransformation products
For five of the products of TAK-715 (m/z 400.148), sufficient material was obtained to acquire a 1H-NMR spectrum, which together with the LC–MS/MS data allowed structure elucidation. By comparison with the spectra and fragmentation patterns of TAK-715, it was possible to assign the structural modifications for most of the products (Table 1). The details of the 1H-NMR spectra can be found in Table S2 of the ESI. Data on interpretation of the LC–MS* spectra are discussed in more detail elsewhere [32]. The proposed structures of the products are summarized in Figure 3.

TAK-P5 and TAK-P6, both with m/z 416.146, indicate mono-oxygenation at different positions. The integrals and coupling pattern of the 1H-NMR spectrum of TAK-P5 showed that all 12 aromatic hydrogen atoms and the hydrogen atoms of the ethyl moiety were still present (Table S2). The fact that the singlet of the benzylc methyl group was no longer observed, whereas the chemical shifts of the aromatic protons were shifted upfield, indicates that TAK-P5 results from hydroxylation of the benzylc methyl group. For TAK-P6, the 1H-NMR-spectrum indicates hydroxylation at the methylene carbon of the ethyl side chain: the triplet of the terminal methyl group in TAK-715 was changed to a doublet, whereas the quartet of the original methylene group was shifted upfield and showed half the integral. The secondary loss of acetaldehyde in the LC–MS/MS data of TAK-P6 was consistent with hydroxylation at this position.

TAK-P7 (m/z 430.123) was the major metabolite formed by HLM. Its 1H-NMR spectrum showed all aromatic hydrogen atoms and an unchanged ethyl side chain. Similar to TAK-P5, the signal of the benzylc methyl group was not observed, whereas the signals of the

Table 1: Identification and characterization of TAK-715 biotransformation products

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<th>IC50 (nM)</th>
<th>95% Conf. interval</th>
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* tret = retention time in the HRS analysis (Fig. 2A and 2B); n.q., not quantified.
aromatic ring of the benzylic ring were strongly shifted upfield. This, in combination with the LC–MS/MS data, indicates that the benzylic methyl group is metabolized to a carboxylate group, e.g., by further oxidation of TAK-P5 by two successive oxidation reactions (Figure 3).

TAK-P1 (m/z 432.138, consistent with two sequential oxygenation reactions) showed a 1H-NMR spectrum in which both the signals of the benzylic methyl group and the ethyl side chain of TAK-715 were strongly affected, suggesting that this product results from hydroxylation of both alkyl side chains. The coupling pattern and integrals of the signals of the ethyl side chain show that hydroxylation occurred at the methylene group, similar to the changes observed in the spectrum of TAK-P6.

Similarly, the 1H-NMR spectrum of TAK-P2 (m/z 446.138) showed strong changes in the signals of the benzylic methyl group and the ethyl side chain. The signals of the ethyl side chain point to hydroxylation at the methylene position, similar to TAK-P1 and TAK-P6, whereas the signals of the benzylic group showed the same changes as observed in TAK-P7, indicative for formation of a carboxylic acid by sequential oxidations (Figure 3).

For products TAK-P3, TAK-P4 and TAK-P9, no 1H-NMR data could be obtained; no P450 BM3-mutant produced these in sufficient amounts. Thus, no purification was attempted. Structure elucidation for these products, based on the LC–MS/MS fragmentation [32], is summarized in Table 1 and Figure 3. For metabolites TAK-P8 and TAK-P10, which result from single and triple oxygenation reactions, respectively, no useful fragment ions were found in LC–MS/MS.

Biotransformation of TAK-715 appeared to occur almost exclusively at its alkyl side chains. TAK-P5 and TAK-P7 result from sequential oxygenation on the benzylic methyl group. The intermediate aldehyde was not detected, suggesting that it extensively undergoes further oxygenation to TAK-P7 and, to lesser extent, TAK-P3. Although conversion of the alcohols to carboxylates is often catalysed by sequential alcohol dehydrogenase, aldehyde dehydrogenase and oxidase reactions, conversion of TAK-P5 to TAK-P7 appears to be fully catalysed by P450 BM3. So far, only few examples have been described on the multistep oxidation of a methyl group to a carboxylic acid by CYPs. Oxidation of a methyl of the t-butyl moiety of terfenadine and ebastine to their corresponding carboxylic acids fexofenadine and carebastine in HLM was previously shown to be catalysed by CYP3A4 and CYP2J2 [33,34]. More recently, yet unidentified microbial P450s from Streptomyces platensis were also shown to be capable to produce fenofexadine from terfenadine [35]. Similar to our P450 BM3 incubations, no intermediate aldehyde was found, indicating that the conversion of aldehydes to acids by P450s is highly efficient. The availability of biocatalysts for conversion of methyl groups to carboxylic acids is considered of high value because chemical synthesis is laborious, and because incorporation of carboxylic acids in drug molecules might be beneficial for water solubility, might decrease the risk of hERG inhibition [35], and might restrict passage of the blood-brain-barrier.

Combining the product profiles of the different P450 BM3 mutants (Table S1) with structural modifications observed in the products (Figure 3), it can be concluded that oxygenation of the benzylic methyl group is by far the major pathway for all mutants studied. The sum of TAK-P5 and TAK-P7, both only involving modification of the benzylic methyl group, represents from 50 to 100% of the products, dependent on the mutant. Because TAK-P1, TAK-P2 and TAK-P3 might be secondary oxidation products of TAK-P5 and TAK-P7, the total contribution of this pathway might even be underestimated. The most selective mutants, which almost specifically produce the primary product TAK-P5, showed only very low enzyme activity, explaining why no secondary product was observed. Only for mutants M11 V87I and M11 V87F, the change in the product profile might be attributed to changes in substrate orientation, because this is the only mutation which did not show any oxygenation of the ethyl side chain: the benzylic methyl oxygenation appeared to represent 90% of the biotransformation. Mutant M11 L437N showed the highest amount of product TAK-P6, and relatively low levels of TAK-P5 and TAK-P7, which suggests that mutation L437N causes a shift from benzylic methyl hydroxylation to hydroxylation of the ethyl side chain.
Thus be restricted to the uncompromised concentrations. Despite the incomplete binding effects will alter the dose-response curves and are difficult to detect in a plate reader setup. In a HRS setup, these interferences are easily detected and the analysis can be resolved by correcting for the extinction coefficient once product standards are available. Subsequently, different concentrations of TAK-715 and TAK-715 metabolites were analysed using the p38α HRS system in flow-injection mode in order to assess the affinity of each compound. Figure 4 shows the concentration dependence of binding of the individual products and TAK-715 to p38α, after correction for the dilutions intrinsic to the HRS-system. Calculated IC₅₀-values of TAK-715 and its products are presented in Table 1. For both TAK-715 and TAK-P₁, not all data points of the full binding curve were considered in the IC₅₀-determination, but those, which had been compromised by interferences, were excluded. This is neither due to a shortcoming in the assay nor due to experimental error. On the contrary, these are two excellent examples of the power of the HRS platform to resolve interferences. In case of the highly lipophilic TAK-715, peak tailing due to nonspecific binding to materials of the HRS system deformed the Gaussian shape so severely at concentrations higher than 60 nM (5µM injected) that it was not possible to calculate the local concentration in the assay. Also, the baseline for the following injection could not be recovered in a reasonable time. In the case of TAK-P₁, (auto)fluorescence at the assay wavelengths appeared to interfere with the p38α affinity measurement at high concentrations. Due to the different concentration-dependent behaviour of both contributions, e.g., increase of TAK-P₁ (auto)fluorescence and decrease of fluorescence enhancement of the tracer by displacement at increasing TAK-P₁ concentration, a W-shaped peak was obtained whose area no longer reflects the fraction of binding. It is important to note that these phenomena would also affect plate reader based assays, albeit with different manifestations. Adsorption to the well plate surface and a mean signal for positive and negative fluorescence contributions would result from high lipophilicity and auto-fluorescence, respectively. The first diminishes the actual concentration of the binder while the latter results in an underestimation of the negative signal height. Both effects will alter the dose-response curves and are difficult to detect in a plate reader setup. However, in a HRS setup, these interferences are easily detected and the analysis can thus be restricted to the uncompromised concentrations. Despite the incomplete binding curves, the IC₅₀-values and 95% confidence intervals can still be estimated for TAK-715 and TAK-P₁ by fitting the data points by the same fitting procedure as was used for the other compounds with complete binding curves. The IC₅₀ obtained for TAK-715, 71 nM is consistent with the value observed previously with this platform [19]. As shown in Figure 4, for three of the products, TAK-P₁, TAK-P₅, and TAK-P₇, the binding curves obtained were shifted to higher concentrations, compared to TAK-715. The largest shift was observed with TAK-P₇. This product showed an IC₅₀-value of 1500 ± 203 nM which is a 20-fold lower affinity than TAK-715. With IC₅₀-values of 460 ± 165 nM and 310 ± 33 nM, respectively, TAK-P₁ and TAK-P₅ showed 5- and 7-fold decreased affinity. Interestingly, TAK-P₆ showed high affinity (IC₅₀-value of 38 ± 4 nM). However, due to the large standard error of the IC₅₀-value of TAK-715, the difference in affinity of TAK-P₆ is not statistically significant. For TAK-P₂, no binding curve was obtained, indicating that this product completely lacked affinity to p38α. These measurements indicate that oxygenation of the benzyl methyl group appears to be detrimental for affinity to p38α. The fact that TAK-P₇ shows a very low affinity, whereas TAK-P₂ shows no affinity at all, indicate that the carboxylic acid strongly disturbs binding to p38α. These results are consistent with these results of Miwatashi et al. which showed that modifying the benzyl methyl group of TAK-715 by other substituents, or by positioning the methyl group to the ortho- or para-position, in all cases led to a strong loss in affinity towards p38α [23]. Apparently, the benzyl methyl group at the meta-position of TAK-715 is essential for its affinity. This is further confirmed by the high affinity of TAK-P₆ in which only the ethyl side chain is hydroxylated. Again, this seems consistent with the study of Miwatashi et al. [23] which showed that modification of the thiazole-2-ethyl moiety of TAK-715 to other alky1 substituents (methyl, propyl) or a large substituent such as 4-methylsulfonylphenyl only showed little effect on p38α affinity.

Conclusion

The results of the present study show that the combination of a catalytically diverse set of P450 BM3 mutants, as a toolbox to diversify a drug, and a HRS-systems capable of rapid screening for affinity to p38α kinase is a promising platform to generate potential novel lead compounds which might have improved physicochemical properties (by improved water solubility) and desired pharmacological properties. As exemplified by TAK-715 as model compound, the P450 BM3 mutants were able to produce almost all metabolites produced by human liver microsomes at significant levels. Furthermore, profiling the effect of structural modifications introduced by P450 on p38α kinase affinity gives valuable information on which structural elements are critical and which are non-critical for affinity. This is an essential tool in the construction of pharmacophore models and the lead optimization process. In addition, it would reduce the necessity of synthesizing large lead libraries by the medicinal chemist. Finally, our results confirm that biocatalysis with CYPs can provide compounds with improved pharmacological and/or physicochemical properties compared to the lead itself which are not easily accessible by classic organic synthesis. This approach can be considered as an extra tool, next to the chemical synthesis in the typical medicinal chemistry approach.

Acknowledgement

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References


17. F. Verhaar, A. A. van der Doelen, J. F. Smits, W. M. Blanksteijn and G. J. Zaman, Inhibition of Wntβ-secretase signaling by p38 MAP kinase inhibitors is explained by cross-reactivity with casein kinase 10c, Chem., 2011, 18, 485-494.


### Supplementary Material

**Figure S1**: Preparative HPLC-UV (254 nm) chromatogram of mixture of biotransformation products obtained by large-scale incubation of TAK-715 with P450 BM3 mutant M11.

### Table S1: Percentage of conversion and ratio between the biotransformation products of TAK-715 incubations with human liver microsomes (HLM) and 33 mutants of P450 BM3a.

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*a. All values represent averages of three individual experiments; RSDs were always less than 10%*  
*b. [M+H]+-values as determined by accurate-mass measurements by HR-MS.*
Table S2: '?'-NMR-spectra of TAK-715 and major biotransformation products formed by P450 BM3 M11.

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<td>1.45-1.48 (3H,t)</td>
<td>1.61-1.63 (3H,d)</td>
<td>1.64-1.65 (3H,d)</td>
<td>1.45-1.48 (3H,t)</td>
<td>1.62-1.64 (3H,d)</td>
<td>1.44-1.49 (3H,t)</td>
</tr>
<tr>
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<td>3.09-3.13 (2H,q)</td>
<td>5.08-5.12 (1H,q)</td>
<td>5.08-5.12 (1H,q)</td>
<td>3.09-3.14 (2H,q)</td>
<td>5.08-5.12 (1H,q)</td>
<td>3.08-3.14 (2H,q)</td>
</tr>
<tr>
<td>Hc</td>
<td>2.33 (3H s)</td>
<td>- 4.9° (s)</td>
<td>- 4.9° (s)</td>
<td>2.32 (3H s)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hd</td>
<td>7.19-7.26 (3H,m)</td>
<td>7.19-7.26 (3H,m)</td>
<td>7.64-7.76 (1H,d)</td>
<td>7.35-7.39 (3H,m)</td>
<td>7.18-7.25 (3H,m)</td>
<td>7.58-7.62 (1H,d)</td>
</tr>
<tr>
<td>He</td>
<td>&quot;</td>
<td>&quot;</td>
<td>7.43-7.46 (1H,t)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>7.40-7.43 (1H,t)</td>
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<td>-</td>
<td>&quot;</td>
<td>7.98-8.00 (1H,d)</td>
</tr>
<tr>
<td>Hg</td>
<td>7.34 (1H,s)</td>
<td>7.51-7.54 (3H,m)</td>
<td>8.18 (1H,s)</td>
<td>7.51-7.54 (3H,m)</td>
<td>7.34 (1H,s)</td>
<td>8.14 (1H,s)</td>
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<tr>
<td>Hh</td>
<td>6.95-6.96 (1H,q of d)</td>
<td>7.00-7.01 (1H,q of d)</td>
<td>7.00 (1H,s)</td>
<td>6.98-6.99 (1H,q of d)</td>
<td>6.97-6.98 (1H,q of d)</td>
<td>6.95-6.96 (1H,q of d)</td>
</tr>
<tr>
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<td>8.23-8.24 (1H,d)</td>
<td>8.25-8.26 (1H,d)</td>
<td>8.82-8.23 (1H,d)</td>
<td>8.22-8.23 (1H,d)</td>
<td>8.21-8.22 (1H,d)</td>
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<tr>
<td>Hj</td>
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<td>8.30 (1H,d)</td>
<td>8.33 (1H,d)</td>
<td>8.29 (1H,d)</td>
<td>8.31 (1H,d)</td>
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</tr>
<tr>
<td>Hk</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hl</td>
<td>7.94-7.96 (2H,d)</td>
<td>7.94-7.95 (2H,d)</td>
<td>7.94-7.95 (2H,d)</td>
<td>7.94-7.96 (2H,d)</td>
<td>7.94-7.95 (2H,d)</td>
<td>7.94-7.95 (2H,d)</td>
</tr>
<tr>
<td>Hm</td>
<td>7.51-7.54 (2H,d of d)</td>
<td>7.51-7.55 (2H,d of d)</td>
<td>7.51-7.54 (2H,d of d)</td>
<td>7.51-7.54 (2H,d of d)</td>
<td>7.51-7.54 (2H,d of d)</td>
<td>7.51-7.54 (2H,d of d)</td>
</tr>
<tr>
<td>Hn</td>
<td>7.59-7.62 (1H,t)</td>
<td>7.59-7.62 (1H,t)</td>
<td>7.58-7.60 (1H,t)</td>
<td>7.59-7.62 (1H,t)</td>
<td>7.59-7.62 (1H,t)</td>
<td>7.58-7.62 (1H,t)</td>
</tr>
</tbody>
</table>

* Chemical shift and integral of benzylic protons could not be determined accurately due to interference with solvent signal.
Chapter 3.3

Comparison of (bio-) transformation methods for the generation of metabolite-like compound libraries of p38α MAP kinase inhibitors using high-resolution screening

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Abstract

Four hydrophobic p38α mitogen-activated protein kinase inhibitors were refluxed with 7.5% hydrogen peroxide at 80°C and irradiated with visible light in order to generate more hydrophilic conversion products. The resulting mixtures were assessed in a high-resolution screening (HRS) platform, featuring liquid chromatographic separation coupled in parallel with a fluorescence enhancement based continuous-flow affinity bioassay towards the p38α mitogen-activated protein kinase and with high-resolution (tandem) mass spectrometry on an ion-trap–time-of-flight hybrid instrument. The results were compared with similar data where chemical diversity was achieved by means of electrochemical conversion or incubation with either human liver microsomes or cytochrome P450s from Bacillus megaterium (BM3s). In total, more than 50 conversion products were identified. The metabolite-like compound libraries studied are discussed in terms of the reactions enabled, the retention of affinity, and the change in hydrophilicity by modification, in summary the ability to generate bioactive, more hydrophilic potential lead compounds. In this context, HRS is demonstrated to be an effective tool as it reduces the effort directed towards laborious synthesis and purification schemes.

1. Introduction

Despite the efforts in, for example, combinatorial chemistry, drug discovery still relies mainly on the utilization of purified compounds for biological affinity or activity testing, due to the limitations of classical high-throughput assays [1]. However, almost every synthetic or biosynthetic Endeavour initially produces a mixture of compounds mainly because of side reactions. As a consequence, significant effort is directed towards optimization of synthesis [2] and purification [3] schemes for discovery compounds. An approach to overcome this bottleneck is the use of high-resolution screening (HRS), which is based on the bioaffinity assessment of individual compounds in a mixture [4] instead of requiring pure compounds or yielding a summed response like in high-throughput assays. By assessing all reaction products of a (parallel) synthesis, optimization and purification efforts can be directed towards active products without having to make a pre-selection.

HRS relies on a combination of separation, mostly liquid chromatography (LC), and hyphenated bioassays. The technology has matured in recent years by gradual improvements in stability and reproducibility as well as by the integration of mass spectrometry (MS) for structure elucidation [4]. We recently developed an HRS platform for the contemporary drug target p38α mitogen-activated protein kinase (p38α) which was thoroughly validated, enabling us to simultaneously assess the structure and affinity towards p38α of individual small molecules in a mixture. p38α is a key node in the cellular response to inflammatory stimuli and has thus been proposed as a drug target for therapy of chronic inflammatory diseases like psoriasis, rheumatoid arthritis or Crohn’s disease [6]. Many high-affinity p38α inhibitors have been created and some of them have advanced as far as clinical phase III in the drug development pipeline [7, 8]. Current p38α inhibitors are often very lipophilic, which negatively influences their solubility and bioavailability [9].

We investigated modifications of known p38α inhibitors by various means aiming to improve their physicochemical properties, which are pivotal aspects in steering bio-availability, while retaining high affinities. Oxidative metabolism can decrease the lipophilicity of the inhibitors and may result in their target affinity [10]. Therefore, techniques used for producing metabolites or metabolite-like compounds may present a promising route to lead libraries with improved physicochemical properties. Next to the increase in hydrophilicity, for example by hydroxylation, these bioactive metabolites may have more selectivity as well [11, 12].

Therefore, we studied several methods that have been used to produce more hydrophilic metabolites, in order to generate metabolite-like lead libraries of conversion products (CPs) from different known p38α inhibitors. Chemical oxidation with hydrogen peroxide (H₂O₂), which is generally used to simulate the influence of molecular oxygen on drugs during long term storage [13, 14], may yield similar products as metabolic reactions, though not by the same mechanisms [15]. Irradiation of drugs with intense visible light (Light), also applied in stability testing, was investigated as well [13]. This approach might not be as promising for the generation of metabolite-like compounds, but Light can initiate photochemical reactions, which possibly modify the scaffold of the molecule, resulting in new active core structures [14, 16]. Electrochemical conversion (EC) has been shown to be able to reproduce certain metabolic reactions, especially N- and O-dealkylation or P- and S-oxidation [17]. HRS data on EC generated libraries of CPs of p38α inhibitors have been reported earlier [18]. An interesting biosynthetic approach is the use of genetically engineered bacterial variants of certain metabolic enzymes, e.g., cytochrome P450s from Bacillus megaterium, especially one called BM3 [19]. These BM3s can be engineered to be highly regio- and stereo-selective [20] as well as for specific product profiles [21]. The library generation of CPs for the p38α inhibitor TAK-715 by means of BM3 mutants has been reported elsewhere [21]. In vitro metabolism simulation by human liver microsomal incubations (HLM) was investigated and compared with the other methods in order to additionally explore the usefulness of HRS in selecting suitable methods for metabolite synthesis in safety testing [22].

This manuscript is part of and actually concludes a larger study. Here, we present the structure and affinity profiles of the lead libraries of CPs of the p38α inhibitors produced with H₂O₂ and Light. In addition, these new results are critically compared to previously reported data, obtained with the other modification methods, with respect to the reactions enabled, the retention of affinity, and the change in hydrophilicity by modification, based on the results of our HRS platform. This enabled us to establish an initial qualitative Structure-Activity Relationship (SAR), which in turn allowed us to implement these methods as toolbox in the generation of metabolite-like lead libraries and judge the potential of their products as lead molecules. Thereby, we show that the implementation of the HRS platform together with a variety of modification methods is likely to create a effective lead optimization process as it reduces the effort directed towards laborious synthesis, purification and testing schemes.

2. Materials and Methods

2.1 Materials

The human recombinant p38α mitogen-activated protein kinase as well as its inhibitors DMP111 (1-(6-chloro-5-(QR,5S)-4-(4-flurofenyl)-2,5-dimethylpiperezine-1-carboxyl)-3aHindol-3-yl)-2-morpholinooethane-1,2-dione), SB203580 (4-[4-(4-flurofenyl)-2-(4-methylsulfinylfenyl)-1H-imidazol-5-yl]pyridine) [23], BIRB796 (N-[3-(3-butyl)-1-(4-methylphenyl)-1H-pyrazol-5-yl]-N-[4-(2-[4-morpholino]ethoxy]-1-naphthalenyl]-urea) [24], and TAK-715 (N-[4-[2-Ethyl-4-(3-methylphenyl)-5-thiaziolyl]-2-pyndinyl]benzamide) [25] come from various sources. Human liver microsomes (HLM) pooled from different individual donors were obtained from BD Gentest TM (San Jose, CA, USA; Cat. No. 452161) and contained 20 mg/mL protein. Methanol (LC-MS grade) and a 30% hydrogen peroxide solution were purchased from Biosolve (Valkenswaard, the Netherlands) and J.T. Baker (Deventer, Holland), respectively. Formic acid was obtained from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) at the highest purity available. Water was generated with a Milli-Q purification system (Millipore, Amsterdam, Netherlands).

2.2 Chemical oxidation

The p38α inhibitors were oxidized by refluxing (ca. 80°C) with hydrogen peroxide. Incubation times were 105 min for DMP111, 180 min for SB203580, and 300 min for TAK-715 (refer to Section 1 of the electronic supplementary material (ESM)). Sampling was done by means of a syringe from a three-neck flask via a septum. The reaction solutions were diluted...
from 1 mM stock solutions in methanol to a solvent composition of 75% aq. hydrogen peroxide and 25% MeOH. They contained an optimized hydrogen peroxide concentration of 7.5% (w/w) and an inhibitor concentration of 100 µM. Control incubations under reflux conditions containing no hydrogen peroxide were included to assess the influence of thermal degradation.

2.3 Photochemical modification
Photochemical modification (Light) was induced by irradiating aliquots of an inhibitor with intense light of the visible range (>310 nm [16]) at room temperature. The exclusion of UV-wavelengths is expected to result in more specific reactions and prevent advanced decomposition. The light was generated by a 150W Xenon lamp model L21 equipped with an AEG transformer. The reaction solutions, diluted from 1 mM stock solutions in methanol to a solvent composition of 75% water and 25% MeOH, were placed at a distance of 15 cm from the lamp. The concentrations of the inhibitors were 100 µM for DMPIP and SB203580 and 10 µM for TAK-715 (see ESM Section 1), respectively. Incubation times were 120 min for DMPIP, 300 min for SB203580, and 30 min for TAK-715 in Light (refer to ESM Section 1). The use of closed Duran glass vessels excluded light of the UV range (<310 nm [16]) and hindered evaporation. Samples were taken with a syringe. To protect the environment from UV radiation, the whole setup was shielded with aluminium foil. Control incubations, which assessed the contribution of heat generated by the Xenon lamp, were prepared by wrapping the samples in aluminium foil to exclude irradiation.

2.4 Microsomal incubations
In order to investigate how closely the products of the modification methods resemble human metabolites, human phase I metabolism was simulated in vitro by incubation with human liver microsomes (HLM). Phase I oxidative metabolites were generated by HLM and cofactor NADPH using a modified version of a protocol described elsewhere [26]. In brief, the reaction mixtures were prepared in 50 mM potassium phosphate buffer (pH 7.4) including 5 mM magnesium chloride. The incubation mixtures contained 100 mM p38α inhibitor, 2 mM/L human liver microsomes and 6 mM NADPH, and were incubated for 2 h at 37°C. Constant availability of NADPH was ensured by a regenerating system of 5 mM glucose-6-phosphate and 5 U/mL glucose-6-phosphate dehydrogenase and by adding 10% (v/v) of a 10 mM NADPH solution in the above mentioned phosphate buffer after 30, 60 and 90 min. The reactions were stopped by addition of ice-cold acetonitrile 2:1 (v:v). The samples were subsequently centrifuged at 16,000 × g for 5 min at 4°C. The supernatants were taken, freeze-dried and stored at –20°C. For the HRS analysis, the samples were re-dissolved in a 30% aqueous methanol solution, providing 10-fold higher concentrations.

2.5 HRS analysis
Analysis of structure and affinity towards p38α was conducted with an HRS platform developed earlier [5]. The HRS platform consisted of two LC-20 AD pumps, two LC-10 AD pumps, a SIL-20 AC autosampler, a CTO-20 AC and a CTO-10 AC column oven, an RF-10AXL fluorescence detector, an SPD-AD UV/VIS detector, a CBM-20A controller, and an ion-trap–time-of-flight mass spectrometer equipped with an ESI source, all products of Shimadzu (s Hertogenbosch, the Netherlands). In short, 10 µL (100 µL for TAK-715 in Light) of the mixture of CPs were separated on a Symmetry C18 column (2.1 x 100 mm, 3.5 µm particles; Waters, Milford, MA, USA) at 40°C and a flow rate of 113 µL/min. LC solvents included water, methanol and formic acid in the ratios 99% / 1% / 0.01% for solvent A and 1% / 99% / 0.01% for solvent B. Eluents were eluted with several gradients optimized per compound and per modification method (see ESM Section 2). The eluent was split, one part being directed to the electrospray ionization high-resolution (tandem) mass spectrometer (HR-MS²) on the Shimadzu ion-trap–time-of-flight mass spectrometer, another constantly mixed with the bioassay reagents and detected by the fluorescence detector. Details on the HR-MS² conditions can be found elsewhere [27].

Figure 1: HRS chromatograms of A) TAK-715 in H₂O₂ and B) DMPIP in Light. The top trace is the fluorescence chromatogram (bioaffinity trace); affinity towards p38α is observed as a negative peak. The bottom traces are the extracted ion chromatograms (EIC) for the different CPs. The colors of the EICs match their corresponding tags (CP numbers). For the m/z values of the EICs, refer to the CPs in A) Table 2 and B) Table 1. Top and bottom chromatograms are aligned to correct for void volume differences. In this way, structure and affinity data can be correlated [18].

3. Results
This paper integrates the new data on CPs generated by Light and H₂O₂ with data generated by other conversion methods. In this sense, it is part of an and summarizes and concludes a larger study [5, 18, 21, 27]. Additionally, it provides a critical comparison of the different methods and summarizes the role of HRS in the study. The conversion of four p38α inhibitors (see Tables 1 to 4 for their structures) with five different modification strategies (EC, Light, H₂O₂, BM3, and HLM) results in a very large and complex data set. Therefore, interpretation of the HR-MS² spectra of the four parent drugs and the structure
elucidation of the more than 50 CPs found (see Tables 1 to 4) was reported separately [27]. Here, we first report the results on the compound libraries of CPs generated using H.O2, Light, and HLM. Proposed structures of the CPs are given in Section 3 to 6 of the ESM. Next, we review and compare the results from the five compound modification strategies in terms of the reactions enabled, the retention of affinity, and the change in hydrophilicity by modification, and thus the ability to generate valuable lead compounds, with potentially improved efficacy, selectivity, and importantly bioavailability.

The HRS platform assesses in parallel the bioaffinity of the CPs by an on-line post-column modification, and thus the ability to generate valuable lead compounds, with potentially improved efficacy, selectivity, and importantly bioavailability.

Table 1: Summary of the conversion products of DMPIP generated with the five applied modification methods. The profile groups (A to D) used in structure elucidation are circled in grey [24]. Isomeric modifications were attributed to the individual profile groups. For structure proposals for these CPs, see ESM Section 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>p38 affinity</th>
<th>RRT</th>
<th>m/z</th>
<th>Delta</th>
<th>Modification</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>X</td>
<td>1.00</td>
<td>541.204</td>
<td>x</td>
<td>x</td>
<td>EC</td>
</tr>
<tr>
<td>CP431 O</td>
<td>0.57</td>
<td>431.148</td>
<td>-C,H,F</td>
<td>Dealkylation of A and de-</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>CP433 O</td>
<td>0.84</td>
<td>433.165</td>
<td>-H,F</td>
<td>Dealkylation of A</td>
<td>BM3</td>
<td></td>
</tr>
<tr>
<td>CP472 X</td>
<td>0.84</td>
<td>472.164</td>
<td>-C,H,N</td>
<td>Amide hydrolysis</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>CP501 O</td>
<td>0.74, 0.67</td>
<td>501.170</td>
<td>-C,H</td>
<td>Double dealkylation in B</td>
<td>EC, H2O</td>
<td></td>
</tr>
<tr>
<td>CP505 ?</td>
<td>0.88</td>
<td>505.226</td>
<td>-HCl</td>
<td>New 5-ring B and C</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>CP523A,C O</td>
<td>0.63 (A), 0.69 (C), 0.67 (D)</td>
<td>523.235</td>
<td>-H,O</td>
<td>Exchange of Cl for H in C and OH in B</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>CP523B, D</td>
<td>0.67 (B), 0.72 (D)</td>
<td>523.235</td>
<td>-</td>
<td></td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>CP525 O</td>
<td>0.80</td>
<td>525.170</td>
<td>-CH3</td>
<td>Methyl loss from B</td>
<td>EC</td>
<td></td>
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<tr>
<td>CP529 O</td>
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<td>529.165</td>
<td>-CH3, O</td>
<td>All modifications in B</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>CP537 ?</td>
<td>0.88</td>
<td>537.252</td>
<td>-HCl</td>
<td></td>
<td>Light</td>
<td></td>
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<tr>
<td>CP539 X</td>
<td>1.22, 1.39</td>
<td>539.187</td>
<td>-H2</td>
<td>Dehydrogenation in B</td>
<td>EC, Light</td>
<td></td>
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<tr>
<td>CP541A ?</td>
<td>0.88</td>
<td>541.204</td>
<td>x</td>
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<td></td>
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<tr>
<td>CP541B X</td>
<td>0.96</td>
<td>534.212</td>
<td>x</td>
<td></td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>CP541C X</td>
<td>1.34</td>
<td>557.197</td>
<td>+O</td>
<td>OH in B</td>
<td>H2O, H3O+</td>
<td></td>
</tr>
<tr>
<td>CP557A X</td>
<td>0.75</td>
<td>557.197</td>
<td>+O</td>
<td>Oxygenation in D</td>
<td>EC</td>
<td></td>
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<tr>
<td>CP557C - 1.10</td>
<td>571.212</td>
<td>N-oxide in C</td>
<td>HLM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP559 X</td>
<td>0.80</td>
<td>559.213</td>
<td>+H,O</td>
<td>Water addition to C or D</td>
<td>Light</td>
<td></td>
</tr>
<tr>
<td>CP571 O</td>
<td>1.08</td>
<td>571.212</td>
<td>+CH,O</td>
<td>Methoxylation in A or B</td>
<td>EC</td>
<td></td>
</tr>
<tr>
<td>CP573 ?</td>
<td>0.88</td>
<td>573.226</td>
<td>+CH,O</td>
<td></td>
<td>Light</td>
<td></td>
</tr>
</tbody>
</table>

X: affinity; O: no affinity observed; ?: unclear, mostly due to co-elution; -: not measured

3.3 Oxidation with hydrogen peroxide

First, the hydrogen peroxide concentration was optimized by testing 3.0%, 7.5%, and 15% (v/v) at a reflux temperature of ca. 80°C to increase reaction speed [31]. With 3.0%, little conversion was achieved, whereas 15% resulted in undesired advanced decomposition of the molecule. As the controls showed that temperature increase alone did not result in significant side reactions, refluxing with 7.5% hydrogen peroxide was applied. The conversion efficiency, measured as the reduction in the parent compound peak area by LC–UV, is ca. 50% after 180 min for SB203580, 30% after 300 min for TAK-715 and 90% after 105 min for DMP (see ESM Section 1). BIRB796 was not included in this part of the study, because initial experiments indicated that the compound was hydrolysis, due to the prevailing acidic conditions, is the main reaction pathway.

3.3.1 DMPIP

The conversion of DMPIP ([M+H]+ with m/z 541.204, CP501 C17H15N2O2S) by hydrogen peroxide (see Table 1) produces two abundant products, both with increased hydrophilicity. The major product CP557A (C11H17N2F2O7S), containing a hydroxylation in the B part (cf. Table 1), is the only one showing affinity, although significantly lower than DMPIP. Whereas MS and UV responses are 2 to 10 times higher, respectively, the affinity response is at least 30 times lower. For the minor product CP501 (C11H15CIFN3O2S), a double dealkylation product of the piperazine ring, no affinity signal is observed.

3.3.2 TAK-715

In treatment of TAK-715 ([M+H]+ with m/z 400.228, CP571 C24H19N2O2S) with hydrogen peroxide, a large number of minor products is yielded which almost exclusively have increased hydrophilicity (see Table 2 and Figure 1A). The strongest UV and MS signal comes from the amide hydrolysis product CP296 (C14H11N2S) which is probably responsible for the first and highest of the product affinity peaks (1A-a) and is also among the most hydrophilic products (RRT 0.35). At least 8 different mono-oxygenated CPs (CP416D to L, C24H17N2O1S) can be distinguished by reweighting time varying largely in hydrophilicity (RRTs range from 0.56 to 1.20). Additionally, two doubly-oxygenated compounds (CP432C and D, C24H17N2O3S) are observed at RRT 0.57 and RRT 0.61. These and other CPs (Table 2) are discussed in more detail in ESM Section 4. At RRTs between 0.5 and 0.8, overlapping affinity peaks are observed (1A-b, 1A-e, and 1A-g), indicating that some of the mono-hydroxylated and possibly some of the double-oxygenated products have affinity for p38α. Due to the high complexity of this sample (Table 2 and Figure 1A), separation is insufficient for exact affinity assignment. Only CP432D and CP416GC can be clearly attributed to 1A-b, 1A-e, and 1A-g, respectively, as the onset of both affinity peaks cannot be attributed to any other CP. However, the width of 1A-b, 1A-e, and 1A-g suggests that there are other co-eluting CPs with affinity. As this study is conducted under a screening paradigm, any further attempt to achieve sufficient separation, thus allowing additional structure identification by MS and more precise affinity assignment, was considered to be out of its scope. Finally, 1A-g
Table 2: Summary of the conversion products of TAK-715 generated with the four applicable modification methods. Individual carbon atoms, at which modifications take place, are numbered. For structure proposals for these CPs, see ESM Section 4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>p38 affinity</th>
<th>RRT</th>
<th>m/z</th>
<th>Delta</th>
<th>Modification</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>X</td>
<td>1.00</td>
<td>400.149</td>
<td>x</td>
<td>x</td>
<td>Light</td>
</tr>
<tr>
<td>CP279</td>
<td>?</td>
<td>1.14</td>
<td>279.096</td>
<td>-C₂H₅NO</td>
<td>Benzamide loss and dehydrogenation</td>
<td>Light</td>
</tr>
<tr>
<td>CP294A</td>
<td>?</td>
<td>0.37</td>
<td>294.107</td>
<td>-C₃H₅O</td>
<td>Amide hydrolysis and dehydrogenation</td>
<td>Light</td>
</tr>
<tr>
<td>CP294B</td>
<td>O</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP296</td>
<td>O (Light, HLM), X (H₂O₃)</td>
<td>0.75, 0.35, 0.35</td>
<td>296.123</td>
<td>-C₃H₅O</td>
<td>Amide hydrolysis</td>
<td>Light, HLM, H₂O₃</td>
</tr>
<tr>
<td>CP312</td>
<td>X</td>
<td>0.21</td>
<td>312.117</td>
<td>-C₃H₅O</td>
<td>Amide hydrolysis and OH</td>
<td>HLM</td>
</tr>
<tr>
<td>CP312B</td>
<td>?</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP384</td>
<td>X</td>
<td>0.91</td>
<td>384.172</td>
<td>-S +O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP398A</td>
<td>?</td>
<td>1.04</td>
<td>398.134</td>
<td>-H₂</td>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>CP398B</td>
<td>O</td>
<td>1.36</td>
<td></td>
<td></td>
<td>Ring formation between carbon-9 and carbon-20</td>
<td>Light</td>
</tr>
<tr>
<td>CP398C</td>
<td>?</td>
<td>1.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP400</td>
<td>O</td>
<td>1.18</td>
<td>400.149</td>
<td>x</td>
<td>Isomer of TAK-715</td>
<td>Light</td>
</tr>
<tr>
<td>CP414</td>
<td>?</td>
<td>1.04</td>
<td>414.128</td>
<td>-H₂ +O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP416A</td>
<td>X</td>
<td>0.80</td>
<td>416.144</td>
<td>+O</td>
<td>OH at carbon-14</td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP416B</td>
<td>X</td>
<td>0.83</td>
<td></td>
<td></td>
<td></td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP416C</td>
<td>O</td>
<td>0.87</td>
<td></td>
<td></td>
<td></td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP416D</td>
<td>?</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP416E+F</td>
<td>?</td>
<td>0.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP416G</td>
<td>X</td>
<td>0.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP416H+I</td>
<td>?</td>
<td>0.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP416J</td>
<td>O</td>
<td>0.87</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP416K</td>
<td>?</td>
<td>1.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP418L</td>
<td>?</td>
<td>1.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP430A</td>
<td>X</td>
<td>0.64</td>
<td>430.123</td>
<td>-H₂ +2O</td>
<td>OH at carbon-2 or-3 and aldehyde at carbon-14</td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP430B</td>
<td>X</td>
<td>0.84</td>
<td></td>
<td></td>
<td>Carboxylic acid at carbon-14</td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP432A</td>
<td>X</td>
<td>0.58</td>
<td>432.139</td>
<td>+2O</td>
<td>OH at carbon-2 and carbon-14</td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP432B</td>
<td>X</td>
<td>0.70</td>
<td></td>
<td></td>
<td>Two OH in ethyl group</td>
<td>(BM3), HLM</td>
</tr>
<tr>
<td>CP432C</td>
<td>?</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP432D</td>
<td>X</td>
<td>0.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP446</td>
<td>O</td>
<td>0.62</td>
<td>446.121</td>
<td>-H₂+3O</td>
<td>Carboxylic acid at carbon-14 and OH at carbon-2 or-3</td>
<td>BM3, HLM</td>
</tr>
<tr>
<td>CP448</td>
<td>X</td>
<td>0.44</td>
<td>448.138</td>
<td>+3O</td>
<td></td>
<td>HLM</td>
</tr>
</tbody>
</table>

* X: affinity; O: no affinity observed; ?: unclear, mostly due to co-elution;

3.1.3 SB203580

The major CP of SB203580 ([M+H]+ with m/z 378; C₂₁H₂₇FN₅OS) is the S-oxidation product CP394C (C₂₁H₂₇FN₅OS⁺). No affinity peaks were observed because these are masked in the bioaffinity trace indicated the presence of another CP, not initially recognized in the MS data, corresponding to CP384 (C₁₉H₁₇FN₂O₂⁺) in which sulphur has been exchanged for oxygen. CP384 only generated a very minor peak in the TIC, and as this sulphur-oxygen exchange reaction was not expected, an EIC trace was initially not generated either. This is a good example of the power of the on-line post-column affinity assay [4]: a low-abundance compound with relatively high affinity, missed in UV or MS detection, will be detected by the affinity detection. Thus, the HRS platform is in a positive way biased towards binders.

Table 3: Summary of the conversion products of SB 203580 generated with the four applicable modification methods. Individual carbon atoms, at which modifications take place, are numbered. For structure proposals for these CPs, see ESM Section 5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RRT</th>
<th>m/z</th>
<th>Delta</th>
<th>Modification</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>1.00</td>
<td>378.108</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>CP243</td>
<td>1.26</td>
<td>243.094</td>
<td>-C₃H₅NS</td>
<td>Sulfide replacement in CP305</td>
<td>Light</td>
</tr>
<tr>
<td>CP289</td>
<td>1.58</td>
<td>289.081</td>
<td>-C₃H₅N</td>
<td>Sulfide to thioether in CP305</td>
<td>Light</td>
</tr>
<tr>
<td>CP305</td>
<td>1.16</td>
<td>305.077</td>
<td>-C₃H₅N +O</td>
<td></td>
<td>See ESM Section 5</td>
</tr>
<tr>
<td>CP316</td>
<td>1.18</td>
<td>316.125</td>
<td>-C₃H₅OS</td>
<td>Sulfide replacement</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP321</td>
<td>1.53</td>
<td>321.072</td>
<td>-C₃H₅N +2O</td>
<td>Methyl hydroxylation in CP305</td>
<td>Light</td>
</tr>
<tr>
<td>CP362</td>
<td>1.44</td>
<td>362.113</td>
<td>-O</td>
<td>Sulfide to thioether</td>
<td>Light</td>
</tr>
<tr>
<td>CP376</td>
<td>0.60</td>
<td>376.113</td>
<td>-F +H₂O</td>
<td>-HF + H₂O</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP392</td>
<td>0.68</td>
<td>392.108</td>
<td>-F +H₂O + O</td>
<td>S-oxidation</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP394A</td>
<td>0.76</td>
<td>394.103</td>
<td>+O</td>
<td>Aromatic OH</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP394B</td>
<td>0.80</td>
<td></td>
<td></td>
<td>Aromatic OH</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP394C</td>
<td>1.05, 1.10,</td>
<td>&quot;</td>
<td>&quot;</td>
<td>S-oxidation</td>
<td>HLM, H₂O₃</td>
</tr>
<tr>
<td>CP394D</td>
<td>1.12</td>
<td></td>
<td></td>
<td>&quot;</td>
<td>N-oxidation</td>
</tr>
<tr>
<td>CP394E</td>
<td>1.36</td>
<td></td>
<td>&quot;</td>
<td>OH in pyridine ortho to N</td>
<td>HLM, H₂O₃</td>
</tr>
<tr>
<td>CP410A</td>
<td>0.83</td>
<td>410.098</td>
<td>+2O</td>
<td>Aromatic OH (CP394A) and S-oxidation</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP410B</td>
<td>0.88</td>
<td></td>
<td></td>
<td>Aromatic OH (CP394B) and S-oxidation</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP410C</td>
<td>1.29</td>
<td></td>
<td>&quot;</td>
<td>S- and N-oxidation</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP410D</td>
<td>1.43</td>
<td></td>
<td>&quot;</td>
<td>OH in pyridine ortho to N</td>
<td>H₂O₃</td>
</tr>
<tr>
<td>CP424A</td>
<td>1.37</td>
<td>424.115</td>
<td>+O +CH₂O</td>
<td>Both in fluorophenyl ring</td>
<td>Light</td>
</tr>
<tr>
<td>CP424B</td>
<td>1.44</td>
<td></td>
<td>&quot;</td>
<td>OH at carbon-14, OCH₃ in pyridine ring</td>
<td>Light</td>
</tr>
</tbody>
</table>
by strong auto-fluorescence as indicated by positive fluorescence peaks throughout the bioaffinity trace. Next to CP394C, four other mono-oxygenation isomers and four different double-oxygenated CPs are observed (Table 3). The latter CPs result from a combination of S-oxidation (CP394C) with each of the other mono-oxygenation reactions. Their hydrophilicity nicely follows the hydrophilicity trend of their respective mono-oxygenation CPs. These and other CPs (Table 3) are discussed in more detail in ESM Section 5.

3.2 Photochemical modification
The conversion efficiency for the photochemical modification (Light) is ca. 20% after 300 min for SB203580, 90% after 300 min for TAK-715 and 70% after 120 min for DMPIP (determined by LC–UV: see ESM Section 1). BIRB796 was also not included in this part of the study, as initial experiments yielded only products of advanced decomposition.

3.2.1 DMPIP
DMPIP ([M+H]+ with m/z 541; C27H26ClF5N5O14) in Light yields a number of minor products (Table 1). CP539 (C27H26ClF5N5O13), the product of a dehydrogenation in the B part (cf. Table 1), shows the highest affinity and the strongest UV response. As expected, this modification increases the lipophilicity of the molecule (RRT 1.39). While the UV response of CP539 is only slightly more than 10% of the residual substrate, the height of the affinity peak is almost 75% of that of DMPIP. Furthermore, three minor affinity signals are observed (Figure 1B). The shoulder (1B-II) at the beginning of the substrate affinity peak (1B-SO) is induced by CP541B (C27H26ClF5N5O13, an isomer of DMPIP). Peak 1B-II might result from up to four co-eluting compounds, being another substrate isomer CP541A (C27H26ClF5N5O13), a dechlorination and ring formation product (CP505, C27H26ClF5N4O13), a product showing the addition of methanol (CP573, C27H26ClF5N5O13), and CP537 (C27H25F5N4O5) combining the modifications of CP505 and CP573. The structure elucidation of these four CPs was hindered by their low abundance and limited fragmentation in MS² (27). Peak 1B-II seems to correlate to a product of (at least apparent) water addition (CP559, C27H25ClF5N5O14). For more information and additional CPs, see ESM Section 3.

3.2.2 TAK-715
TAK-715 ([M+H]+ with m/z 400; C37H38N6OS5) in Light produces three major (CP398A and B and CP400) and two minor (CP279 and CP296) CPs (Table 2), none of which show affinity, although affinity of CP398A and CP279 may be masked by that of TAK-715. The fact that the amide hydrolysis product (CP296, C36H36N6S5) does not show affinity here, as opposed to H0, is most likely a concentration effect. Some other CPs (Table 2) are discussed in ESM Section 4. Except for CP296, all products were less hydrophilic than the substrate.

3.2.3 SB203580
SB203580 ([M+H]+ with m/z 378; C21H19FN5O9S) in Light forms only minor products. Again, no affinity peaks were observed (see 3.1.3). Some CPs (Table 3) are discussed in ESM Section 5. CPs of Light show exclusively decreased hydrophilicity.

3.3 In vitro metabolism
HLM incubations of the four kinase inhibitors will be discussed except for TAK-715 which have been described elsewhere [21], but the results are included in Table 2. This addresses the question whether the other modification methods produce real metabolites in addition to interesting CPs, because HLM incubations are still the gold standard in cell-free in vitro metabolism [32].

3.3.1 DMPIP
The incubation of DMPIP with HLM results in only one minor mono-oxygenation metabolite CP557C (C28H31ClF5N5O14), produced by N-oxidation of the C ring (cf. Table 1).

3.3.2 BIRB796
The in vitro metabolism of BIRB796 ([M+H]+ with m/z 528/265; C16H16N6O15) resulted in a more complex mixture (Table 4). Two mono-oxygenation metabolites, CP544A and CP544B (C16H16N6O14) were observed at RRTs of 0.84 and 0.87, respectively. These result from hydroxylation of the A part (CP544A) or of the B part (CP544B) (cf. Table 4). Other low-abundance mono-oxygenation products were present at higher RRT. Furthermore, two co-eluting double-dealkylation metabolites were detected. CP502 (C16H15N6O15) and CP458 (C16H15N6O15) show the loss of ethyne and dihydrofuran, respectively, from part D. Additionally, three metabolites are partly co-eluting at RRTs of 1.27 and 1.25. One of these less hydrophilic metabolites is the hydroquinone CP415 (RRT 1.27, C16H15N6O15) resulting from loss of the ethyl-morpholino group by O-dealkylation. Additionally, CP1052 (C16H15N6O15) is a dimerization product with two additional bonds in the C and/or D part. CP459 (C21H19N5O9S) results from oxidative deamination of the CPs. These and other CPs (Table 3) are discussed in more detail in ESM Section 5.

3.3.3 SB203580
The in vitro metabolism of SB203580 ([M+H]+ with m/z 378; C16H15N6O15) has been investigated by Henklova et al. [33]. However, in addition to the reported S-oxidation metabolite (CP394C, C16H16FN5O14), we observed a minor metabolite showing N-oxidation instead (CP394D, C16H15FN5O14) (Table 3). This additional finding is probably due to the higher sensitivity of HR-MS², which significantly increases the signal-to-noise ratio when analysing complex samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>p38 affinity</th>
<th>RRT</th>
<th>m/z²</th>
<th>Delta</th>
<th>Modification</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>X</td>
<td>1.00</td>
<td>264.653(2)</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CP230</td>
<td>O</td>
<td>0.95</td>
<td>230.165(1)</td>
<td>-C2H3NO</td>
<td>AB</td>
<td>EC</td>
</tr>
<tr>
<td>CP273</td>
<td>O</td>
<td>1.04</td>
<td>273.171(1)</td>
<td>-C2H3NO</td>
<td>AB+CO-NH3</td>
<td>EC</td>
</tr>
<tr>
<td>CP413</td>
<td>X</td>
<td>1.22</td>
<td>413.199(1)</td>
<td>-C2H5NO</td>
<td>quinoneimine</td>
<td></td>
</tr>
<tr>
<td>CP415</td>
<td>X</td>
<td>1.19, 1.27</td>
<td>415.215(1)</td>
<td>-C2H4NO</td>
<td>ether hydrolysis</td>
<td>EC,HLM</td>
</tr>
<tr>
<td>CP458</td>
<td>-</td>
<td>0.90</td>
<td>229.632(2)</td>
<td>-C2H5O</td>
<td>2x N-dealkylation</td>
<td>D</td>
</tr>
<tr>
<td>CP459</td>
<td>-</td>
<td>1.25</td>
<td>459.242(1)</td>
<td>-C2H7N</td>
<td>alcohol for morpholino</td>
<td>D</td>
</tr>
<tr>
<td>CP502</td>
<td>-</td>
<td>0.90</td>
<td>251.645(2)</td>
<td>-C2H7</td>
<td>O-and N-dealkylation</td>
<td>D</td>
</tr>
<tr>
<td>CP544A</td>
<td>-</td>
<td>0.84</td>
<td>272.650(2)</td>
<td>+O</td>
<td>OH in A or B</td>
<td>HLM</td>
</tr>
<tr>
<td>CP544B</td>
<td>-</td>
<td>0.87</td>
<td>&quot;</td>
<td>&quot;</td>
<td>OH in A</td>
<td>HLM</td>
</tr>
<tr>
<td>CP1052</td>
<td>-</td>
<td>1.25</td>
<td>526.285(5)</td>
<td>2x bonds</td>
<td>in part C or D</td>
<td>HLM</td>
</tr>
</tbody>
</table>

* X: affinity; O: no affinity observed; -: not measured

Charge state (n): [M+nH]⁺
morpholino group, exchanging the latter for a hydroxyl group. Most metabolites show relatively low UV peak areas, except for the peak that corresponds to the three co-eluting, less hydrophilic metabolites (CP1415, CP459, and CP1052). This peak has about 20% of the UV area of the residual substrate peak.

4. Discussion

4.1 Comparison of compound modification methods

The results of the five modification methods (H₂O₂, Light, EC, HLM, and BM3) are discussed and compared in different ways. First, we look at the structures of the CPs generated and correlate them to affinity. The parallel setup of on-line post-column affinity assay and MS² enables a straightforward structure-affinity comparison and significantly reduces the risk of errors in the correlation. Hydrophilicity, as a physicochemical parameter of major importance in modern drug discovery [9], is used in addition to affinity in order to assess the quality of the products as potential new leads. To this end, CPs are marked as more hydrophilic (RRT<0.90), unchanged (0.90≤RRT≤1.10) or less hydrophilic (RRT>1.10) compared to the substrate based on their RRTs. The clogP values of the substrates were calculated with ChemBioDraw version 12 to be 5.8 for BIRB796 (logP 5.2 [34]), 6.5 for TAK-715, 2.7 for SB203580 and 2.3 for DMPIP. Finally, the most interesting CPs as seen from various viewpoints are highlighted. The ability to generate human relevant metabolites using the modification methods is also briefly discussed.

4.1.1 DMPIP

An overview of the CPs yielded from DMPIP is given in Table 1 and ESM Section 3. In general, there is little overlap in the products of DMPIP between the different modification methods, except the double N-dealkylation product CP501, generated by H₂O₂ as well as EC, and the dehydrogenation product CP539, appearing in Light and EC: CP433 was almost exclusively produced by BM3, whereas a similar product CP431 with an additional double bond in the B ring was produced by the EC. Light is the only method inducing chlorine hydroxylation exchange (CP523A to D), isomerisation (CP541A to D) and the addition of water (CP559). In contrast, methyl abstraction (CP529) and amide hydrolysis (CP472) are not observed in EC. HLM all products are oxygenated compound (CP557A to C). However, all three modifications are found in different parts of the molecule and while EC produces hydroxylation, HLM results in N-oxidation.

Seven of the 22 CPs showed bioaffinity, ten did not and for another five the analysis was inconclusive. From correlating structure and affinity, we can conclude that the fluorobenzyl ring is more important for affinity than the morpholino group, as removal of the former in CP433 and CP431 leads to complete affinity loss while upon loss or modification of the latter in CP472 and CP557B affinity is retained. Furthermore, the dimethyl substitution at the piperazine ring seems crucial as all dealkylations such as the loss of a methyl group in CP525, and the hydrocarbon losses in CP501 and CP529 delete affinity. Dehydrogenation even produces the high-affinity compound CP539. Interestingly, the exchange of the chlorine for a hydrogen atom has a huge negative effect on affinity. Additionally, at least two of the three substrate isomers (CP541A to C) are binders. These could be promising modified scaffolds for further lead library diversification.

Many CPs showed an improvement in physicochemical properties: fifteen of the 22 CPs were more hydrophilic, five had similar hydrophilicity and only two were less hydrophilic than the substrate. Interestingly, the only metabolite from HLM was less hydrophilic than the substrate.

The most interesting compound of this series is probably CP539 as it showed by far the highest product affinity peak in all DMPIP derived mixtures. Whether this was due to higher abundance and/or higher affinity than other products cannot be determined. Unfortunately, CP539 shows decreased hydrophilicity, but the reduction of stereochemical complexity might be advantageous. Furthermore, CP472 and CP557A are of special interest because they show bioaffinity and increased hydrophilicity. Additionally, CP472 is simpler and the hydroxyl group of CP557A might result in more specific interactions with the target, for example through hydrogen bonding.

4.1.2 TAK-715

The CPs related to TAK-715 are listed in Table 2 and ESM Section 4. Electrochemical conversion was not achieved under the tested conditions [18]. In the other methods, generally, four types of modifications were observed. Dehydrogenation was found in all methods, whereas oxygenation occurred in HLM, BM3 and H₂O₂ and the hydrolysis of the amide bond in Light, H₂O₂ and HLM. A combination of oxygenation and dehydrogenation was also frequently observed. Additionally, in Light, a loss of the complete benzamide moiety as opposed to simple hydrolysis is observed. This is especially interesting as a similar reaction is observed in collision-induced dissociation (CID) of the protonated molecule [27]. HLM and BM3 show the most similar profile, HLM having slightly more products, including those with amide hydrolysis [21]. However, H₂O₂ also yields many products with similar modification to HLM and BM3, but with much more isomeric mono-oxygenated products. Additionally, only H₂O₂ and HLM show a combination of amide hydrolysis and oxygenation. Finally, Light shows three products not observed with the other methods. Twelve of the 32 CPs showed bioaffinity, six did not and for another fourteen the analysis was inconclusive. The higher number of CPs results in a higher uncertainty in the affinity determination, mainly owing to co-elution, especially of the mono-oxygenation isomers in H₂O₂. HLM and BM3 showed the most impressive results in generating CPs with affinity, but not at the same concentration in Light. Correlating structure and affinity indicates that many of the oxygenation products retain affinity, even after multiple reactions (up to three were observed). CP416B even has similar affinity as TAK-715 [21]. Loss of the phenyl ketone group by amide hydrolysis seems to have some effect on affinity, because the affinity of CP296 is only observed at high concentration in H₂O₂, but not at the lower concentration in Light. This discrepancy also result from (possible) affinity of co-eluting CP294A and/or CP312B. Dehydrogenation by ring formation in CP398B exerts a strong negative influence on affinity, whereas formation of a carbonyl group at the aromatic methyl group in CP430A and CP430B only results in lower affinity, e.g., CP430B affinity is about 20 times lower than TAK-715 [21]. The TAK-715 isomer (CP460) also does not show affinity.

By generating many oxygenated CPs, HLM, BM3 and H₂O₂ also produced a compound library with many more hydrophilic CPs. In Light, this was only the case for CP296. In total, 23 of the 32 CPs were more hydrophilic, whereas six had decreased hydrophilicity. The latter were equally contributed by Light and H₂O₂, but accounting for as much as 60% of products in Light and only 16% in H₂O₂.

A rather unusual reaction for BM3 is the formation of a carboxylic acid from the aromatic methyl group in CP430B [21]. Though possessing the right elemental composition, the aldehyde CP414 from H₂O₂ is unlikely to be the missing link between the alcohol CP416A and the carboxylic acid CP430B, as under aqueous conditions an aldehyde would be transformed into the carboxylic acid by a strong oxidizing agent like hydrogen peroxide. The most interesting CP is CP416B, which not only shows significantly increased hydrophilicity but also similarly high affinity as TAK-715.

4.1.3 SB203580

The CPs of SB203580 which were produced are summarized in Table 3 and ESM Section 5. No affinity signals for CPs of SB203580 have been observed in any of the measurements, possibly due to the strong auto-fluorescence of SB203580 at the assay wavelength. Oxygenation was introduced by all modification methods. Next to the major metabolite, i.e., the S-oxidation product CP394C, also reported by Henklova et al [33], many other mono-
and double-oxygenated isomers have been generated. In Light, the oxygenation was often accompanied by additional reactions, and in H_{2}O by dehalogenation. Interestingly, the CP profile generated by EC is most similar to HLM, whereas H_{2}O produces all products of HLM but also many more, such as CPs involving loss of the sulfonyl group (CP316). The most uncommon reactions are again observed with Light, such as S-reduction to the thioether CP362, methoxylation in CP424A and CP424B, and the breakdown of the imidazole ring to CP305 and its related products.

Generating more hydrophilic CPs was less successful. Only six of the 19 CPs were more hydrophilic. Interestingly, all six more hydrophilic compounds were from H_{2}O, as were six of the less hydrophilic compounds.

### 4.1.4 BirB796

CPs of BirB796 were only generated by EC and HLM. Initial tests indicated that BM3, H_{2}O, and Light were not suitable to produce interesting compound libraries. Ten CPs observed are listed in Table 4 and ESM Section 6. Only the two hydroxylation isomers in HLM are more hydrophilic. There is little overlap between HLM and EC CPs. Where HLM mainly catalyses hydroxylation and N- and O-dealkylation, EC is mostly active around the urea function. However, two CPs generated by both methods are important in metabolism studies: the reactive quinoneimine CP413 and its corresponding unstable hydroquinone CP414. Both CPs showed affinity. They are the main products in EC whereas CP415 seems to be among the most abundant CPs in HLM. The fact, that CP413 is only detectable in HLM close to the limit of detection, does not mean that CP413 is not an important metabolite. On the contrary, quinoneimines may react with free cysteines in proteins to form covalent drug-protein adducts [35]. They can be detected as SH adducts in specially designed experiments [36] which were outside the scope of this investigation.

### 4.2 Conversion products as metabolite standards

Another interesting aspect of this study is the possibility to generate metabolites formed using HLM by other conversion methods (H_{2}O, EC, Light, and/or BM3s). As this may yield an alternative to conventional organic synthesis. The synthesis of standards for metabolic analysis often is a bottleneck in the drug discovery phase. It might be assumed that BM3, as another biotransformation method, shows the closest resemblance of product profiles. As discussed in detail elsewhere [21], the HLM and BM3 profiles for Tak-715 were very closely related for some mutants. Herein, the flexibility of the BM3 approach is crucial. However, H_{2}O was found to produce an HLM metabolite of Tak-715 which BM3 did not. For DMPIP, the HLM metabolite CP557C was not produced by BM3 or any other method, although H_{2}O and EC did give other oxygenation products. For SB203580, H_{2}O was the only method to produce both HLM metabolites, the S- and the N-oxidation product, although in addition to a large number of other compounds. EC worked very well here as its only major product was the main metabolite CP394C. For BirB796, HLM can only be compared to EC. At first glance, the overlap seems quite limited. However, EC produces one (possibly two) very important metabolites as main product(s), CP413 and CP415, which, being reactive species might be very interesting for the toxicology of BirB796 [37]. Another interesting observation in this study was that, contrary to general expectation, many HLM metabolites with decreased hydrophilicity were observed. Only for Tak-715, all metabolites were more hydrophilic, while for DMPIP and SB203580 exclusively less hydrophilic metabolites were observed. This underlines the importance of taking into account metabolites with decreased hydrophilicity when developing analytical methods for metabolite identification.

Though BM3 shows the most possibilities to be tuned for the production of specific metabolites, the inability of H_{2}O to produce many metabolite-like isomers can also be exploited. With adequate fractionation technology, it might even be more advantageous to produce many standards simultaneously with H_{2}O, especially at a stage when the true metabolite identities are not yet confirmed. EC confirmed its value in the generation of reactive metabolites, exemplified by CP413 and CP415, but is also useful for the generation of stable metabolites, as is shown for SB203580. Thus, the combination of all four methods represents a valuable toolbox for the generation of metabolite standards. HRS contributes to the application of this toolbox by rapidly identifying the method(s) of choice for a specific substrate. The standards can then be produced by up-scaling and purification which can also be monitored with the HRS system.

### 5. Conclusion

Four modification methods were investigated for their potential to produce metabolite-like compounds. The main goal was to find CPs with retained affinity and increased hydrophilicity, which may act as lead compounds in further drug discovery. Therefore, the data evaluation emphasized the reactions occurring, the affinity of the CPs towards p38a and the hydrophilicity of the CPs. First of all, it can be concluded that the HRS system is very useful for the described type of study. An efficient analysis of the modifications introduced via the different methods was enabled. Affinity and identity of the CPs could be assessed simultaneously. However, ambiguity remains in some cases. Limited chromatographic resolution may hinder identification of the CP with affinity, especially in very complex mixtures, such as with Tak-715 in H_{2}O and DMPIp in Light (Figure 1). Whereas many conversion reactions like oxygenation, dehydrogenation and amide hydrolysis are readily identified, limited fragmentation (and/or sensitivity) may hinder structure elucidation of CPs, for example in distinguishing between N-oxidation and aromatic hydroxylation, or between different oxygenated isomers. Direct quantitation of CPs from a mixture is still missing in the HRS technology, as is also true for early metabolic profiling unless radioactive detection is applied [38]. However, the qualitative affinity data in combination with the MS structure analysis were sufficient to achieve an initial structure-activity assessment. The combination of hydrophilicity, affinity and structure efficiently starts the characterization of CPs concerning their usefulness as potential new lead compounds. The strong link between the three properties, ensured by the HRS platform, increases the confidence in all results. All this is possible, and was in fact achieved, without fractionation, directed synthesis or extensive sample preparation. Mostly, mixtures of related compounds are analysed in a single chromatographic run. This leads to an extremely efficient collection of a multitude of relevant data on the prospective lead compounds. In earlier work, we demonstrated that this provides a very efficient basis to focus purification efforts on favourable compounds which can then be followed up by in-depth structural characterization and further biological testing [21]. Although H_{2}O and Light converted all tested substrates, the CPs produced did not always show affinity or increased hydrophilicity. EC failed to convert Tak-715. After pre-screening, BM3, was only applied to Tak-715 and DMPip. In terms of the number of products, successful combinations of modification method and substrate were EC/DMPip, Light/DMPip, Light/SB203580, H_{2}O/Tak715, H_{2}O/SB203580, BM3/Tak715, HLM/Tak715 and HLM/BirB796. Retention of affinity is more or less evenly distributed among the tested conversion methods. There is no single one which exclusively or predominantly forms affinity compounds. With 50-70% of the related CPs showing retention of affinity, the screening of DMPip and Tak-715 was quite successful in this respect. All methods, except HLM, are reasonably well scalable. H_{2}O only requires low cost reagents and Light and EC minor investment in apparatus. BM3, however, necessitates expensive purified enzymes and cofactors. Of course, this comparison applies to the analytical scale. It might be very different at large-scale production. High cost and limited availability, apart from ethical considerations, in combination with a low catalytic activity exclude HLM as an alternative for biosynthesis. H_{2}O yielded mostly more hydrophilic CPs, with the exception of some N-oxides and the general tendency for less hydrophilic CPs in SB203580. H_{2}O also produced more
isomeric species than the other methods. Although this increases the chance of finding affinity compounds, it reduces the method’s value in the production of specific CPs. BM3 incubation seems less generally applicable but, when applicable, is effective in generating more hydrophilic CPs with high affinity. Furthermore, it is possible to tune these enzymes for the production of either complex mixtures with a variety of CPs or specific CPs [21].

EC is complementary to the other methods by producing unique products, as is most obvious in DMPiP and BIRB796. It shows its strength in the conversion of BIRB796 where it enables the analysis of the reactive quinoneimine [18]. While it is reasonable to assume that CP metabolite is also produced by HLM, it is the low matrix content and direct analysis capabilities featured by the EC which readily allowed structure and affinity analysis of the reactive species. In this respect, the complexity and reactivity of the sample matrix increase in the order Light<EC<BM3<HEL. A more complex matrix will not only hinder the analysis, especially of reactive products, but also increases the difficulty of purification, if desired at a later stage. Therefore, EC is the most promising method to study relevant reactive species. Unfortunately, the CPs from EC and Light show no general tendency towards more hydrophilicity, although such CPs are still frequently observed. However, Light opens up a wider range of reaction pathways which often results in at least some unique products. Structural changes induced in Light are not easily identified by HR-MS as Light displays the unique possibility to induce strong structural isomerism even in the core of the molecule. Inducing structural changes to the molecules core, while maintaining affinity (see CP541B and CP541C of DMPiP), can lead to a new scaffold for optimization which could be desirable, for example when selectivity of the old scaffold has proven insufficient. Therefore, the four modification methods are highly complementary with regard to the substrates converted, the reactions observed, the isomers formed from similar reactions, the affinities in different substrates and the tendencies for more hydrophilic CPs. All of them created interesting CPs that have the potential to be further explored as lead compounds.

6. Acknowledgements

This research was performed within the framework of project D2-102 “Metabolic stability assessment as new tool in the Hit-to-Lead selection process and the generation of new lead compound libraries” of the Dutch Top Institute Pharma. Vanina Rea is acknowledged for her contribution to the BMD3 data.

References

Supplementary Material

Section 1: Experimental details

The optimization of temperature and hydrogen peroxide concentration for H2O2 and of the incubation time for H2O2 and Light was monitored on an LC–UV setup. This consisted of two LC-20AD pumps, an SIL-20AC autosampler, an SPD-20AD UV/VIS detector, a CBM-20A controller (all products of Shimadzu, ‘s Hertogenbosch, The Netherlands) and a Mistral column oven (Spark Holland, Emmen, The Netherlands). With an injection volume of 10 μL, chromatographic separations were achieved on a Symmetry C18 column (2.1 x 100 mm, 3.5 μm particles; Waters, Milford, MA, USA) at 40°C and a flow rate of 113 μL/min. LC solvents included water, methanol and formic acid in the ratios 99% / 1% / 0.01% for solvent A and 1% / 99% / 0.01% for solvent B. The following gradient was used: 2 min isocratic at 20% B, then a linear gradient to 90% at 18 min, washing at 90% for 4 min (22 min) and afterwards re-equilibration at 20% from 23 min to 30 min. UV detection was achieved at 280 nm.

10 μL injections of samples with a 100 μM starting concentration were used in all H2O2 and Light conversion experiments with the exception of TAK-715 in Light. TAK-715 was not soluble at 100 μM without the presence of hydrogen peroxide which decreased the pH and thereby most probably increased the polarity of TAK-715 by protonation in H2O2. Therefore, the starting concentration of TAK-715 in Light was decreased to 10 μM. Consequently, the injection volume was increased to 100 μL to yield similar concentrations in the LC–UV and HRS analysis.

The substrate consumption was quantified by LC–UV using calibration curves of the inhibitors at concentrations between 5 and 100 μM for DMPIP and SB203580. For TAK-715, the injected concentrations were between 50 and 100 μM or between 1 and 10 μM in the calibration for H2O2 and for Light, respectively. All calibration solutions were freshly prepared in the same way as the respective sample solutions. This was important, because significant matrix effects were observed when comparing calibration curves with and without hydrogen peroxide. The injection volumes for the calibration solutions were the same as for the respective conversion experiments.

For optimization of the incubation time, aliquots were taken at different time points.

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<tr>
<th>Time (min)</th>
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<tr>
<td>0, 10, 20, 30, 45, 60, 75, 90, 105 and 120</td>
<td>DMPIP; every 30 min from 0 to 300 min for SB203580; and every 15 min from 0 to 105 min for DMPIP; every 30 min from 0 to 180 min for SB203580; and at 0, 75, 120, 165, 210, 255 and 300 min for TAK-715.</td>
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<td>3, 6, 9, 12, 15, 20, 25 and 30</td>
<td>TAK-715.</td>
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This yielded the optimized incubation times and the corresponding conversion rates described in the manuscript. Both calibration solutions and samples were measured triplicate. In H2O2, the standard deviation was generally higher than in Light, probably because photochemical reactions are started and stopped much more reliably by introducing and removing the light source than the oxidation reaction are by heating and cooling.

Section 2: Overview chromatographic conditions

In the measurements of samples from EC, BM3 and HLM (TAK-715 only), an Xbridge C18 column (100×2.1 mm with 3.5 μm particles; Waters, Milford, MA, USA) was used. For samples from H2O2, Light and HLM (DMPIP, SB203580 and BIRB796) experiments, a Symmetry column (same dimensions and manufacturer) was applied. Because the results reported here originate from several independent studies, different gradient programs were used. A flow rate of 113 μL/min and a column temperature of 40°C were applied in all cases.
Table S1: Overview of the binary solvent gradients used in different parts of the study, depending on conversion method and starting compound investigated.

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Used for: EC, BM3 (DMPIP) and HLM (DMPIP, SB203580, BIRB796)

Section 3: Structure proposals of DMPIP related CPs

The structure elucidation by LC–MS is described in detail elsewhere [1]. For the convenience of the reader, structure proposals are drawn here on the basis of that analysis. In this way, the structure-activity discussion can be followed more visually. Note that the structures of the corresponding molecules are depicted, not the ions detected by LC–MS.

Additionally, CP537 and CP573, which were not yet discussed, are examined.

CP505

CP523A and C

Four isomers CP523A to D (C_{28}H_{32}FN_{4}O_{5}) are produced by dechlorination and hydroxylation in Light, none of which show affinity. In contrast to CP523B and D, for CP523A and C the spectral data was insufficient for a structure proposal.

CP523B and D

CP525

CP529

CP537 (C_{29}H_{34}FN_{4}O_{5}) is a combination of the dechlorination and ring formation observed in CSP05 and the addition of methanol found in CP573. The structure elucidation of CP537 was hindered by low abundance and limited fragmentation in MS.

CP541A, B and C

These are isomers of the substrate DMPIP. Even where fragmentation is different among the isomers, interpretation of the fragmentation trees is extremely challenging. Thus, as in this case, structural isomers of the substrate are often not identified by LC–MS.

CP557A, B and C

These are isomers of the substrate DMPIP. Even where fragmentation is different among the isomers, interpretation of the fragmentation trees is extremely challenging. Thus, as in this case, structural isomers of the substrate are often not identified by LC–MS.
This product, showing the addition of methanol (CP573, C$_{29}$H$_{35}$ClFN$_4$O$_5$+), did not provide sufficient fragmentation in MS$^n$ to propose a structure. Amounts in Light, is unclear.

**Section 4: Structure proposals of TAK-715 related CPs**

The structure elucidation by LC–MS$^n$ was described in detail elsewhere [1]. For the convenience of the reader, structure proposals are drawn here on the basis of that analysis. In this way, the structure-activity discussion can be followed more visually. Note that the structures of the corresponding molecules are depicted, not the ions detected by LC–MS$^n$. Additionally, some CPs, which were not yet discussed, are examined.

The low-abundance CPs partially derived from amide hydrolysis are relatively polar and show RRTs similar to CP296. These include CP294A and B, CP310, CP312B and CP328. It is reasonable to assume that these are present as a number of isomers which are not chromatographically resolved.

This low-abundance CP of H$_2$O$_2$ combines three different modifications, being amide hydrolysis, oxygenation and dehydrogenation (m/z 310.102, C$_{17}$H$_{16}$N$_3$O$_3$+).

This low abundant product of amide hydrolysis and double-oxygenation (m/z 328.113, C$_{17}$H$_{18}$N$_3$O$_2$S+ ) is made in H$_2$O$_2$.

In this CP, the sulphur has been exchanged for oxygen (C$_{24}$H$_{20}$N$_3$O$_2$S+). Whether the oxygen occupies the same position in the new molecule is unclear.

A dehydrogenation product (C$_{24}$H$_{20}$N$_3$OS+ ), which cannot be further evaluated due to insufficient LC–MS$^n$ data.

This CP is one of the major products in Light and an isomer of TAK-715 (C$_{24}$H$_{22}$N$_3$OS+).

A product of combined oxygenation and dehydrogenation (C$_{24}$H$_{20}$N$_3$O$_2$S+). As it was produced by H$_2$O$_2$, CP414 is unlikely to be an aldehyde as this would most likely be converted to the carboxylic acid under these strong oxidizing conditions.

Unfortunately, due to extensive co-elution of these seven mono-oxygenation isomers (C$_{24}$H$_{22}$N$_3$O$_2$S+), structure elucidation was not achieved by LC–MS$^n$. As this study was focused on a screening paradigm, further separation was not attempted. However, their RRTs between 0.57 and 0.87 indicate that most of them will be hydroxylated [2].

The RRTs of over 1.1 of these two mono-oxygenation CPs indicate N-oxidation [2].

Unfortunately, due to extensive co-elution of these seven mono-oxygenation isomers (C$_{24}$H$_{22}$N$_3$O$_2$S+), structure elucidation was not achieved by LC–MS$^n$. As this study was focused on a screening paradigm, further separation was not attempted. However, their RRTs between 0.57 and 0.87 indicate that most of them will be hydroxylated [2].

The RRTs of over 1.1 of these two mono-oxygenation CPs indicate N-oxidation [2].
Section 5: Structure proposals of SB203580 related CPs

The structure elucidation by LC–MS$^n$ was described in detail elsewhere [1]. For the convenience of the reader, structure proposals are drawn here on the basis of that analysis. In this way, the structure-activity discussion can be followed more visually. Note that the structures of the corresponding molecules are depicted, not the ions detected by LC–MS$^n$. Additionally, some CPs, which were not yet discussed, are examined.

The CPs marked with an asterisk are related to CP305 for which two alternate structure proposals are given [1]. For the sake of brevity, only one possible form is given for the related CPs.

**CP430B**

**CP432A**

**CP432B**

These two double-oxygenation isomers (C$_{24}$H$_{22}$N$_3$O$_2$S$^+$) did not yield sufficient LC–MS$^n$ data for structure elucidation.

**CP432C and D**

**CP446**

**CP448**

**CP305**

One of the two most abundant CPs (by MS response) in Light.

**CP316**

Interestingly, CP316 (C$_{20}$H$_{15}$FN$_3$+) is similar to a gas phase CID fragment (m/z 315.117, C$_{20}$H$_{14}$FN$_3$+•). The methyl-sulfoxide group has been replaced by hydrogen decreasing polarity.

**CP321**

**CP362**

**CP376**

Resulting from a combination of dehalogenation and mono-oxygenation (C$_{21}$H$_{18}$N$_3$O$_2$S$^+$), this is one of the two most polar CPs derived from SB203580 in H$_2$O$_2$.

**CP392**

Resulting from a combination of dehalogenation and double-oxygenation (C$_{21}$H$_{18}$N$_3$O$_3$S$^+$), this is one of the two most polar CPs derived from SB203580 in H$_2$O$_2$.

**CP394A and B**

These CPs result from aromatic hydroxylation (RRT 0.76 and 0.80).

**CP394C**

**CP394D**

CP394D is an N-oxidation product (RRT=1.24).

**CP394E**

CP394E shows strongly reduced polarity which matches cLogP calculation of an ortho-hydroxylated pyridine [1].

**CP410A**

Combines the S-oxidation of CP394C and the aromatic hydroxylation of CP394A.

**CP410B**

Combines the S-oxidation of CP394C and the aromatic hydroxylation of CP394B.

**CP410C**

Combines the S-oxidation of CP394C and the N-oxidation of CP394D.

**CP410D**

Combines the S-oxidation of CP394C and the aromatic hydroxylation of CP394D.
Section 6: Structure proposals of BIRB796 related CPs

The structure elucidation by LC–MS\textsuperscript{n} was described in detail elsewhere [1]. For the convenience of the reader, structure proposals are drawn here on the basis of that analysis. In this way, the structure-activity discussion can be followed more visually. Note that the structures of the corresponding molecules are depicted, not the ions detected by LC–MS\textsuperscript{n}. Additionally, some CPs, which were not yet discussed, are examined.

BIRB796

This CP is essentially a dimer of two BIRB796 molecules. The two substrate molecules are probably linked by two new bonds resulting in the loss of two times H\textsubscript{2} compared to two full BIRB796 molecules. Both links are found in part C and/or part D.

References
Section 4

Integrated structure elucidation approaches for high-resolution screening

Chapter 4.1

Tandem mass spectrometry study of p38α kinase inhibitors and related substances

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Abstract

The p38 mitogen-activated protein kinase (p38α) is an important drug target widely investigated for therapy of chronic inflammatory diseases. Its inhibitors are rather lipophilic, and as such not very favourable lead compounds in drug discovery. Therefore, we explored various approaches to access new chemical space, create diversity, and generate lead libraries with improved solubility and reduced lipophilicity, based on known p38α inhibitors, e.g., BIRB796 and TAK-715. Compound modification strategies include incubation with human liver microsomes and bacterial cytochrome P450 mutants from Bacillus megaterium (BM3) and treatment by electrochemical oxidation, \( \text{H}_2\text{O}_2 \), and intense light irradiation.

The MS/MS fragmentation pathways of p38α inhibitors and their conversion products (CPs) have been studied in an ion-trap–time-of-flight MS\(^*\) instrument. Interpretation of accurate-mass MS\(^*\) data for four sets of related compounds revealed unexpected and peculiar fragmentation pathways which are discussed in detail. Emphasis is put on the usefulness of HRS-MS\(^*\)-based structure elucidation in a screening setting and on peculiarities of the fragmentation with regard to the analytes and the MS instrument.

In one example, an intramolecular rearrangement reaction accompanied by the loss of a bulky group is observed. For BIRB796, the double-charge precursor ion is used in MS\(^2\), providing a wider range of fragment ions in our instrument. For TAK-715, a number of related compounds could be produced in a large-scale incubation with a BM3 mutant, thus enabling comparison of the structure elucidation by \( \text{H}^1\text{-NMR} \) and MS\(^*\). A surprisingly large number of homolytic cleavages is observed. Competition between two fragmentation pathways involving either the loss of \( \text{CH}_3 \) or \( \text{OH} \) radicals was observed for SB203580 and its conversion products.

Introduction

The mitogen-activated protein kinase p38, especially its isoform p38α, is an important target in drug discovery and development directed at chronic inflammatory diseases like Crohn’s disease and rheumatoid arthritis [1-3]. It is involved in signal transduction pathways through successive activation by phosphorylation [4]. Several p38α inhibitors are in different stages of (pre)clinical drug development [2,5]. Typical examples are TAK-715 [6], BIRB796 [7,8] and SB203580 [9] (see structures in Figures 4-6).

In the past few years, we worked on the development of an integrated platform based on high-resolution screening (HRS) to assist in lead optimization and enhancing lead diversity in drug discovery processes, e.g., involving an on-line bioaffinity assay for steroid metabolites [10]. HRS is the parallel analysis of molecular structure and affinity/activity towards a target protein performed on a mixture of related compounds and assessing both qualities individually for each compound. Therefore, the platform consists of a combination of liquid chromatography and parallel on-line continuous-flow bioactivity/bioaffinity screening and high-resolution mass spectrometry (HRMS) [11]. In this way, simultaneous bioactivity/bioaffinity assessment and (tentative) molecular structure elucidation in mixtures of related compounds is possible.

Recently, we reported on an HRS platform to study p38α binding, which enables high-quality bioaffinity assessment (\( Z=0.8 \); S/N up to 100) [12]. This enables us to investigate mixtures of p38α inhibitors and their related substances, generated by various means of metabolism-like compound conversion. Such an approach can be especially useful for highly lipophilic compounds like kinase inhibitors, as the compound modification may result in more polar, but still bioactive analogues that are commonly more difficult to generate by conventional organic synthesis.

For example, the use of electrochemistry in an on-line combination with the HRS platform resulted in a number of related substances for p38α kinase inhibitors, including unstable and reactive conversion products [13]. Mixtures of related substances can also be generated enzymatically, e.g., biosynthetically using (human) microsomal incubations or by incubation with specific bacterial mutants (BM3) of cytochrome P450s [14]. Alternatively, other (chemical) means such as photochemistry or oxidative reagents can be applied. These different means to create compounds of p38α kinase inhibitors are compared in a separate study, involving assessment of bioaffinity and identity by the HRS platform [15].

This paper focuses on the structure elucidation aspect of the HRS platform with special attention on MS fragment interpretation. The study resulted in a wealth of MS/MS data, not only for the kinase inhibitors itself, but also for their related substances, that is: their conversion products (CPs). The interpretation of these data and the identification of most of the CPs of the typical small-molecule kinase inhibitors TAK-715, BIRB796, SB203580, and DMP1P are discussed in more detail in this paper. Emphasis is on specific pathways, fragments crucial in structure elucidation, and peculiarities of the gas-phase chemistry, and the potential of using an ion-trap–time-of-flight hybrid instrument in a screening setting, where fragmentation methods have to be comprehensive. Some of the applied conversion methods enabled the generation of sufficient material of the related substances in order to confirm the HRMS identification with \( \text{H}^1\text{NMR} \) spectroscopy data.

Materials and methods

Chemicals

The human recombinant mitogen-activated protein kinase p38α isoform and its inhibitors DMP1P (1-[6-chloro-5-[(2R,5S)-4-(4-fluorobenzyl)-2,5-dihydroxypropionamide]-1-carboxy]-3aHindol-3-yl)-2-morpholinoethane-1,2-dione), SB 203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine), BIRB796 (N-[3-[[3-(tert-butyl)-1-(4-methylphenyl)-1H-imidazol-5-yl]-N-[4-[(2-[(4-morpholinyl)ethoxy]-1-naphthalenyl)yl]urea]-N-(4-[(2-ethyl-4-(3-methylphenyl)-5-thiazolyl)-2-pyridinyl]benzamide) were obtained from various sources. Methanol (LC–MS grade) and formic acid (LC–MS grade) were from Biosolve (Valkenswaard, the Netherlands). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Schnelldorf, Germany).

Generation of related substances of p38α kinase inhibitors

Various methods of compound conversion were used. Each of these methods is discussed briefly but has been described in detail elsewhere. Electrochemistry adds (reduction) or removes (oxidation) electrons from the substrate on a surface electrode which results in further reaction for example with the solvent [13]. During incubation with (human) liver microsomes [14,15] or with mutants of cytochrome P450 BM3 [14] the enzymes present catalyse specific reactions like hydroxylation. Oxidation with hydrogen peroxide occurs mainly via reactive oxygen species [15] while photochemical conversion is the result of the excitation of electrons in the substrate by intense visible light irradiation [15].

HRS platform: LC with parallel bioaffinity screening and high-resolution MS

The HRS analysis strategy of bioaffinity and chemical composition of the CPs is conducted with the integrated LC–p38α binding assay/MS platform shown in Figure 1 which has been previously reported [12]. In short, the platform consists of a Shimadzu (‘s Hertogenbosch, the Netherlands) LC–MS system, including two LC-20AD and two LC-10AD isocratic pumps, an SIL-20AC autosampler, a CTO-20AC or a CTO-10AC column oven, an RF-10AXL fluorescence detector, an SPD-AD UV/VIS detector, a CBM-20A controller, and an ion-trap–time-of-flight hybrid mass spectrometer (IT–TOF) for HRMS, operated with an electrospray ionization (ESI) source. The mixtures of CPs were separated in the chromatographic part of the HRS platform on an Xbridge C18 column 100×2.1 mm with 3.5 mm particles (Waters, Milford, MA, USA) at 40°C using a flow rate of 113 mL/min. The following mobile phases were employed: eluent A (1 % methanol, 99 % water, and 0.01 % formic acid); eluent B (99 % methanol, 1 % water, and 0.01 % formic acid). With these, the gradient was constructed as

![Abstract](image-url)
follows: from 0 to 2 min isocratic at 20% B; from 2 to 18 min, linear increase of eluent B from 20% to 90%; from 18 to 22 min, isocratic at 90% B; from 22 to 23 min linear decrease from 90% to 20% of eluent B; from 23 to 30 min re-equilibration to isocratic conditions at 20% eluent B. Because the different modification techniques were assessed in several studies, both gradient profile and stationary phase varied. For example, the reaction mixtures were most complex in the Light and H$_2$O$_2$ incubations, so specialized gradients were used per substrate. An overview about chromatographic conditions can be found in Section 1 of the Supporting Information and the associated text.

The eluent was split post-column in a ratio of 1:9 using 13 mL/min in the bioaffinity detection and 100 mL/min in ESI-HRMS analysis. The p38α binding assay is not described here as the bioaffinity data is irrelevant to the structure elucidation and the discussion of the MS fragmentation. Ionization by ESI was achieved with the following settings: needle voltage 4.5 kV; source heating block and the curved desolvation line temperature 200°C; drying gas pressure 62 kPa; nebulizing gas flow-rate 1.5 L/min. The IT–TOF instrument provides a resolution of ~10,000 (FWHM) in both MS and MS$^n$ modes. External calibration on sodium trifluoroacetate clusters was applied. The mass accuracy achieved for precursor ions and fragments was generally within ±5 ppm; otherwise, the actual mass accuracy obtained for a particular ion is specified. Deviations larger than ±5 ppm can mostly be explained by low abundance or overlap with the isotope pattern of other species.

Full spectra were obtained in the positive-ion mode between m/z 200 and 650. MS$^2$ and MS$^3$ spectra were obtained in data-dependent mode between m/z 100 and 650 with an ion accumulation time of 10 ms and a precursor isolation width of 3 Da. MS, MS$^2$ and MS$^3$ spectra as well as bioaffinity data are collected in a single run of the HRS platform. Where necessary, the collection of MS$^2$ and MS$^3$ spectra was repeated with manual precursor selection in MS$^2$ using the HRS platform with inactive bioaffinity detection. The collision energy was set to 75% for TAK-715 and its products and to 50% for all other compounds. Structure elucidation was based on calculating the elemental composition of precursor and fragment ions from the accurate-mass measurements. Several restrictions were applied to limit the analysis to relevant elemental compositions. For the parent ions, the elemental composition of the substrate plus a reasonable number of atoms which can be added by the respective modification techniques was used as a cut off. Mostly, this is restricted to oxygen, but also carbon and hydrogen were used where for example methoxylation was possible. For the analysis of fragment ions, the restriction criterion applied was the elemental composition of the precursor ion. With these restrictions, most often only one elemental composition was yielded within 5 ppm error. In rare cases, where in spite of the restrictions several elemental compositions were obtained, the false compositions could be easily excluded on basis of carbon-hydrogen-ratio, carbon-nitrogen-ratio and/or the absence of a sensible modification route in the case of the parent ions and/or the absence of sensible neutral losses in the case of the fragments. The structures of the CPs should be viewed as carefully compiled proposals as confirmation by a second, independent analytical technique or through synthesis of standards was out of the scope of this study. An exception is presented by the five purified TAK-715 derived CPs analysed by NMR where such confirmation has been demonstrated.

Where applicable, $^1$H-NMR analysis was performed on a Bruker Avance 500 (Fallanden, Switzerland) at 500.23 MHz.

**Results**

The molecular structure of the major conversion products (CPs) originating from four structurally different p38α inhibitors was elucidated using accurate-mass measurement and MS$^2$ and MS$^3$ data from an IT–TOF MS instrument. These CPs were generated by

![Figure 1](image1.png)

**Figure 1:** Schematic setup of the HRS platform. The analysis of MS fragmentation and the resulting structure proposals of the CPs are the topic of this manuscript. Therefore, the HR-MS part is highlighted because it provides the accurate mass fragmentation data.

![Figure 2](image2.png)

**Figure 2:** MS/MS spectra of the four substrates. The precursor ions are selected in the ion trap within a window of ±1.5 amu around the following m/z values A) m/z 541.2 for DMPIP, B) m/z 378.1 for SB203580, C) m/z 529.3 for BIRB796 and D) m/z 400.1 for TAK-715.
different modification methods, being enzymatic incubation with human liver microsomes (HLM) and a bacterial cytochrome P450 BM3 mutant as well as by chemical treatment using electrochemical oxidation, \( \text{H}_2\text{O}_2 \), and intense light irradiation. The resulting mixtures of CPs were analysed by LC coupled to a continuous-flow p38α enzyme binding assay and parallel HRMS to obtain MS\(^2\) and MS\(^3\) data. The interpretation of MS\(^3\) data is discussed elsewhere [13]. The fragmentation is more readily discussed, if the molecule is divided elsewhere [15].

The structure elucidation of CPs generally proceeds in three steps. First, the MS\(^2\) and MS\(^3\) data of the parent compounds were interpreted in considerable detail, trying to recognize representative fragments to act as profile groups in the molecule [16,17]. In the second step, from a comparison of the elemental composition of the CPs with that of the parent compound, the nature of the modification was established. In the third step, the fragmentation of the CPs was compared to that of the parent to assess modification in identifying the modification, e.g., to differentiate between hydroxylation and \( N \)-oxide formation [18]. In the case of five BM3-generated CPs of TAK-715, a fourth step involved purification of the CPs by preparative LC and subsequent acquisition and interpretation of the \(^1\)H-NMR spectra, guided by the knowledge obtained from the MS\(^3\) data [14]. The data are summarized in the Tables 1 to 4. For most CPs, not all fragments are tabulated, but rather the fragments important for structure elucidation. MS\(^3\) spectra of most of the CPs are included in the Supporting Information. The discussion is not comprehensive as it does not include structure elucidation of all CPs found.

**Structure elucidation of DMPIP conversion products**

The fragmentation of DMPIP (\( m/z \) 541.204, \( \text{C}_{28}\text{H}_{31}\text{ClF}_3\text{N}_2\text{O}_4 \)) has partly been described elsewhere [13]. The fragmentation is more readily discussed, if the molecule is divided into four parts (A to D, see Figure 3) at the main fragmentation sites. The major fragment

![Figure 3: Structure and profile groups of DMPIP as well as proposed structures for the fragment ions.](image-url)
observed in MS² (see Figure 2A) is due to the CD-part (m/z 319.048, C₆H₄ClFN₃O⁺); the complementary AB-part (m/z 223.161, C₅H₄FN⁺) is also observed as well as the C-part (m/z 206.000, C₅H₄ClNO⁺). Two other fragments can be explained by an intramolecular rearrangement of the fluorobenzyl group [19,20] which effect results in the loss of dimethylpiperazine (the B-part, C₆H₄N₂) and the formation of the fragment ion with m/z 427.087 (ACD-C₅H₄ClFNO⁺). The fragment ion with m/z 286.043 (AC-CO-C₅H₄ClFNO⁺) is a secondary fragment of the ACD-ion, involving the loss of both the D-part and CO (see Figure 3). In MS³ of ACD, a fragment ion with m/z 312.024 (AC-H₂-C₅H₄ClFNO⁺, 6 ppm) is formed which is consistent with the loss of part D as morpholine-N-carbaldheyde and thus requires ring formation in the A and/or C part. The two other ions observed in MS³, m/z 284.029 (C₅H₄ClFNO⁺, 6 ppm) and m/z 257.041 (most likely C₅H₄NO⁺, -24 ppm), are probably fragments of this AC-H₂ fragment resulting from the loss of CO and a combination of HF and Cl, respectively. MS² of CD results in the C-ion (m/z 206.000, C₅H₄ClNO⁺). The MS³ spectrum of AC-CD can be readily explained in terms of neutral losses, that is: m/z 258.048 due to the loss of CO, m/z 222.072 due to the loss of CO and HCl, m/z 196.069 due to the loss of CO, HCN, and Cl, and m/z 109.044 due to C₅H₄F⁺ (7 ppm; A-part), but proposing fragmentation routes and fragment structures is more difficult. The DMPIP CPs found with all conversion methods are summarized in detail in Table 1; MS² spectra of most of the CPs are included in the Section 5 of the Supporting Information. In general only a few of the above identified fragments of DMPIP are actually observed for the CPs, especially the CD-ion. CPs generated by EC are discussed in more detail elsewhere [13].

In HLM incubations, DMPIP gave only one minor mono-oxygenated CP (CP557C; m/z 557.195, C₂₀H₁₇F₁₄N₂O⁺). Both the CD- and AC- ions in MS² (m/z 335.045, C₁₈H₁₄ClFNO⁺) and the CD (m/z 321.995, C₁₇H₁₃ClFNO⁺) in MS³ contain the chlorine atom and the fluorobenzyl group. In MS³, the C-ion is observed with an additional water loss (m/z 203.986, C₆H₄ClNO⁺). The water loss and RRT indicate N-oxidation rather than aromatic hydroxylation in the C-ring [18].

In BM3 incubations, DMPIP almost exclusively produced CP433 (m/z 433.165, C₁₇H₁₄ClFNO⁺), consistent with dealkylation of the fluorobenzyl group (loss of A) which could be identified based on accurate mass only.

In H₂O, DMPIP produces two abundant CPs. The major product is CP557A (m/z 557.196, C₂₀H₁₇F₁₄N₂O⁺), which is a mono-oxygenated CP. The MS² spectrum indicates the oxidation must have taken place in the B-part, as the m/z-values for the ACD, CD-, and AC-CD-ions are not changed (see Figure 3). The fragment ion with m/z 376.108 (C₁₈H₁₄ClFNO⁺) results from the loss of both the A-part (CH₂F) and cleavage in the oxidized dimethylpiperazine ring, involving the loss of C₅H₄NO⁺. This ring cleavage, which is not observed for the DMPIP itself, apparently is stimulated by the presence of the extra O at the dimethylpiperazine group. In MS³, this ion shows losses of either C₅H₄N⁺ (57.06 Da) to an ion with m/z 319.052 (C₁₇H₁₃FNO⁺) or of the D-part to an ion with m/z 261.043 (C₁₆H₁₂FNO⁺). This behavior in MS³, the RRT of 0.75, and the absence of losses of H₂O, OH⁻ or O₂ suggest hydroxylation rather than N-oxidation.

The minor product (CP501, m/z 501.170, C₁₇H₁₄ClFNO⁺) was also observed in EC experiments and was identified as resulting from the loss of C₅H₄ from the dimethylpiperazine group [13].

Photochemical conversion of DMPIP yields a product CP539 (m/z 539.187, C₁₇H₁₄ClFNO⁺), also observed in EC experiments, [13] which is due to dehydrogenation of the B part. This interpretation is supported by the increased lipophilicity, and the observation of an unmodified CD fragment in MS² as well as a neutral loss of the unmodified A part as a neutral fragment (m/z 258.048 due to the loss of CO, Cl, and H). Interestingly, the AB fragment (m/z 223.161, C₅H₄FN⁺) is observed with m/z 219.129 (C₅H₄FN⁺) and 220.135 (C₅H₄FN⁺, -10 ppm), thus showing an additional H₂ loss, maybe due to conjugation.

Other photochemical CPs observed include a product (CP559, m/z 559.213, C₁₇H₁₄ClFNO⁺), consistent with the addition of H₂O (Δ +18.001 Da) to the C or D part, and another showing the loss of HCl (CP505, m/z 505.226, C₁₇H₁₄FNO⁻). The latter modification probably involves the generation of a five-membered ring between carbon formerly carrying the chlorine and the B part. At least two isomers are observed of CP523 (m/z 523.236, C₂₈H₂₃FNO⁻), which are consistent with a combination of both modifications. The observation of several isomers already proves that the chlorine is not directly exchanged by a hydroxyl group. Despite significant co-elution, two of the major isomers, CP523B and CP523D, can be identified as loss of Cl from the C-ring and presence of OH in the B-ring. This is concluded from an accordingly modified CD-fragment and similarities to CP557A fragmentation, respectively.

The example of the mono-oxygenated CPs nicely demonstrates the strength of the profilegroup approach [16,17]. The structural isomers CP557A, B and C can be shown to be distinctly modified in part B, D and C, respectively. On top of that, the RRT [16] is very helpful to identify CP557A as a hydroxylation and CP557C as an N-oxidation product. However, if the RRT of the product is close to 1.00, as for CP557B, the latter approach is less helpful. In CP539, the addition of a single double bond remarkably changes the pattern of proton/hydride rearrangement during CID and even leads to the onset of homolytic cleavage of the amide bond. Another interesting change in CID fragmentation, caused by modification, is observed in the CPs characterized by hydroxylation of the B part, namely CP557A and CP523B and D. This modification affects an internal fragmentation of the piperazine ring not observed in any of the other CPs, including DMPIP itself. The interpretation of structural isomers of the substrate remains a challenge as the fragmentation trees are either similar or the changes are extremely difficult to interpret.

**Structure elucidation of SB203580 conversion products**

The fragmentation of SB203580 (m/z 378.108, C₁₇H₁₄FNO₃S⁻) in the IT-TOF is similar to that in the Q-TOF [21]. The only fragment ions observed (see also Figure 2B) are due to the loss of CH₃ (F₃63; m/z 363.084, C₁₄H₁₃FNO₃S⁻) and of SO⁻ (F₃15; m/z 315.117, C₁₃H₁₂FNO₃S⁻). The SB203580 CPs found with all applicable conversion methods are summarized in detail in Table 2; MS² spectra of most of the CPs are included in Section 6 of the Supporting Information.

In H₂O, EC and HLM conversion, the major product formed (CP394C; m/z 394.103, C₁₃H₁₄F₃₆₂₃N₂O₃S⁻) is consistent with transformation of the sulfoxide to a sulfone group [21]. From the unmodified F315 fragment and the absence of F363 (or a corresponding F363+O) (m/z 379.080, C₁₄H₁₃FNO₃S⁺O⁺) alone, S-oxidation cannot be distinguished from CH₃ hydroxylation. However, under Light, a compound with CH₃-hydroxylation was observed for which HClO loss was strongly favored over SO₂CH₃ loss (see CP321 below). Having the fragmentation patterns of both modifications allows to distinguish them. A minor product in HLM and H₂O conversion is another mono-oxygenation product (CP394D). The major fragment (F363+O) excludes both CH₃-hydroxylation and S-oxidation. In MS², the odd-electron ion F363+O is further fragmented by OH⁻ loss (m/z 362.078, C₁₃H₁₄F₁₂N₃O₃S⁻), suggesting N-oxidation [22,23]. This is consistent with the comparably high RRT [18]. The strong preference for the CH₃ loss over OH⁻ loss from the N-oxide suggests that the OH⁻ loss is significantly easier from the odd-electron ion than from the even-electron ion [24-26]. Minor products of H₂O conversion include two mono-oxygenation isomers (CP394A and CP394B), which also show F363+O as major fragment. However, these two species involve hydroxylation at the aromatic system, as F315+O (m/z 331.112, C₁₃H₁₄F₂NO₃S⁻) is observed. The latest eluting isomer (CP394E) shows the same fragmentation pattern, consistent with either a lipophilic aromatic hydroxyl product or an unusually stable N-oxide. cLogP calculations predict a hydroxyl group para to the nitrogen of the pyridine ring results in significantly increased lipophilicity. Four double-oxygenated isomers (m/z 410.098, C₁₇H₁₆F₂NO₅S⁻) were observed in H₂O conversion. The two earliest eluting isomers (CP410A and CP410B) show the loss of SO₂CH₃ to generate F315+O. This indicates a combination of S-oxidation and aromatic
hydroxylation; the RRT are in good agreement with assuming an S-oxidation of CP394A and CP394B. The third isomer (CP410C) shows the loss of either OH (m/z 393.096, C_2H_4FN_2O_3S) or SO CH to F315+O, indicating an N-oxide-S-oxide product, which is also consistent with the RRT, as both modifications increase lipophilicity. F315+O alone does not establish S-oxidation. The fragment might also result from an SOCH loss from m/z 393, in the case of an unmodified sulfoxide group. However, S-oxidation is proven by fragmentation of m/z 393, thus after OH loss, resulting in the loss of the sulfonyl moiety either as SO CH (m/z 314.106, C_2H_4FN_2S), 11 ppm) or as SO CH, leading to F315+O. It is interesting to observe that, because the S-oxidation completely suppresses the CH_3 loss, the OH loss in this case occurs from an even-electron ion. Based on RRT, the last isomer (CP410D) seems to be a combination of the lipophilic aromatic hydroxylation product CP394E and the sulfoxide to thioether. Like for CP410A and CP410B, the only MS^2 fragment is F315+O, thus the nature of the modifications must be the same.

Photochemical conversion of SB203580 results in two major and four minor products. One major product (CP362; m/z 362.113, C_15H_14FN_2O_S) results from the conversion of the sulfoxide into the corresponding thioether. Fragment ions consistent with the loss of CH_3 and CHS confirm this interpretation.

Based on its elemental composition and the assumption that no major rearrangement takes place, the other major product (CP305; m/z 305.077, C_15H_14FN_2O_S) involves a decomposition of the imidazole ring, that is: replacing pyridine and its linking carbon in the imidazole ring by an O-atom (see structure proposals & comments in Section 3 of the Supporting Information). The position of the O-atom is not easy to assess as MS^2 fragments show an arrangement of the N-atoms which is different from the original imidazole ring. Both phenyl rings are observed as a free amide, thus suggesting that carbon-3 and -5, each with an N-atom linked to it, are bridged by the O-atom. However, whether this rearrangement of the modified imidazole ring occurs in the liquid phase during light irradiation or in the gas phase during ionization and/or fragmentation is not clear. Three of the minor products can be considered to be secondary products of CP305. CP243 (m/z 243.094, C_15H_14FN_2O_S) is consistent with the replacement of the methylsulfoxide with H, CP289 (m/z 289.081, C_15H_14FN_2O_S) with conversion of the sulfoxide into the corresponding thioether, whereas CP321 (m/z 321.072, C_15H_14FN_2O_S) involves hydroxylation of the methyl group, as indicated by the loss of H_2C=O to yield the ion with m/z 291.059 (C_15H_14FN_2O_S) rather than the loss of SO CH that would occur after S-oxidation (see CP394C).

Two minor isomeric products (CP424A and CP424B; m/z 424.115, C_15H_14FN_2O_S) result from combined hydroxylation and methoxylation. In MS^2, both CPs generate an ion with m/z 259.088, due to the loss of H_2NOS (H_2C=S(O)=C_2H_4S) indicating that the modifications are either in the pyridine or in the fluorophenyl ring. In the MS^2 spectrum of the earlier eluting CP424A, the ion with m/z 155.052 (C_15H_14F_2O_S) indicates both modifications have occurred in the fluorophenyl ring. A similar fluorophenyl fragment was not observed for other CPs of SB203580. For CP424B, the fluorophenyl fragment is observed with m/z

Table 2: SB 203580 and its conversion products (CPs), showing elemental composition of the CP and the change (Delta) compared to the parent compound, the occurrence of some specific fragments indicated in the text, proposed identification and site of modification (refer to figure 4), relative retention time (RRT) and the technique by which a particular CP is generated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elemental Composition</th>
<th>Other Modification</th>
<th>RRT</th>
<th>Technique</th>
</tr>
</thead>
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<td>X</td>
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<tr>
<td>CP305 and CP316</td>
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<td>X</td>
<td>n.a.</td>
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<tr>
<td>CP305 and CP316</td>
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<td>-SOCH_{3}</td>
<td>X</td>
<td>n.a.</td>
</tr>
<tr>
<td>CP305 and CP316</td>
<td>C_{15}H_{14}FN_{2}O_{S}</td>
<td>-SOCH_{3}</td>
<td>X</td>
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<td>CP362</td>
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<td>X</td>
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<td>X</td>
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<td>X</td>
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<td>-SOCH_{3}</td>
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Figure 4: Structure of SB203580.
123.025 \( (C_2H_FO^-) \) in MS\(^2\). This correlates to a quinone-like structure of this carbocation including carbon-3 and an added oxygen. As the para-position is blocked by fluorine, the aromatic hydroxylation must be ortho to carbon-3, otherwise, there would be no quinone. Since the methoxylation is present in the ion with \( m/z \) 259 but not in the ion with \( m/z \) 123, it must have occurred in the pyridine ring.

Interestingly, the loss of \( SO_2CH\_3 \) indicates \( S \)-oxidation (CP394C) while methyl hydroxylation leads to different fragmentation (CP321), but only because the methyl hydroxylation is more unambiguously interpretable. This means that both structures can be determined without standards when both fragmentation trees are available. CPA24B is one of the rare cases where an aromatic hydroxylation can be linked to a single carbon atom (14a and 14b are chemically identical). The reason is that a very small fragment containing the hydroxylation leads to different fragmentation (CP324), but only because the methyl hydroxylation is more chemically identical. The reason is that a very small fragment containing the hydroxylation leads to different fragmentation (CP324), but only because the methyl hydroxylation is more chemically identical. The reason is that a very small fragment containing the hydroxylation leads to different fragmentation (CP324), but only because the methyl hydroxylation is more chemically identical.

**Structure elucidation of BIRB796 conversion products**

For a discussion on its fragmentation, BIRB796 (\( m/z \) 529.300; \( C_{15}H_{18}N_5O_4^- \)) can be divided into four parts (see Figure 5). Major fragments (see Figure 2C) result from cleavages in the urea moiety, leading to two sets of complementary fragments: the \( AB \)-part and the \( CD \)-part with cleavage on each side of the carbonyl. This results in the fragments \( AB \) with \( m/z \) 230.164 (\( C_{10}H_{17}N_4O_4^- \)), \( AB+CO \) with \( m/z \) 305.143 (\( C_{15}H_{19}N_5O_5^- \)), \( CD \) with \( m/z \) 273.158 (\( C_{12}H_{17}N_4O_4^- \)), and \( CD+CO \) with \( m/z \) 299.138 (\( C_{15}H_{19}N_5O_5^- \)). Both \( AB \) fragments show the loss of the \( B \)-part (\( C_2H_7 \)) to the \( A \)-part, that is, \( m/z \) 200.082 (\( A+CO; C_{10}H_{17}N_4O_4^- \)) and \( m/z \) 174.103 (\( A; C_{10}H_{16}N_4O_4^- \)) (see Figure 5).

In our instrument, BIRB796 also generated a double-charge ion (\( [M+2H]^2+; m/z \) 264.652 (\( C_{13}H_{17}N_5O_4^- \))). The MS\(^2\) spectrum with the double-charge ion as precursor ion partly yields all the fragments observed in the MS\(^1\) of the single-charge ion, but in addition fragment ions with \( m/z \) 414.206 (\( ABC\_2; C_{15}H_{18}N_5O_4^- \)), \( m/z \) 413.197 (\( ABC\_2H; C_{15}H_{19}N_5O_4^- \)), both due to the loss of the \( D \)-part, as well as fragment ions with \( m/z \) 229.156 (\( AB\_2; C_{10}H_{17}N_4O_4^- \)), \( m/z \) 114.092 (\( D; C_{10}H_{16}N_4O_2^- \)), 6 ppm), and \( m/z \) 100.076 (\( D; C_{10}H_9N_4O^- \)) (see Figure 5). It is quite intriguing to observe the formation of pairs of odd-electron and even-electron ions for \( m/z \) 414/413 and for \( m/z \) 229/230 from the double-charge ion. No double-charge fragment ions were observed. In this particular case, the MS\(^2\) on the double-charge precursor ion also enables to get around the low-\( m/z \) cut-off typical of the ion trap: for the double-charge precursor ion with \( m/z \) 264.7, fragment ions with \( m/z \) 100 and 114 are observed, whereas they were not for the single-charge precursor ion with \( m/z \) 528.3. Further MS\(^3\) fragmentation of the various fragment ions is summarized in Section 2 of the Supporting Information. In the MS\(^2\) data of the CPs, not all these fragments are actually observed: \( AB+CO \), \( A+CO \) and \( D \) were observed most frequently. As the fragmentation of the double-charge ion provides better abundance and information of the four profile groups recognized in BIRB796, it was chosen as precursor ion in the structure elucidation of most CPs. The BIRB796 CPs found with all conversion

![Figure 5: Structure and profile groups of BIRB796 as well as proposed structures for the fragment ions in MS\(^2\) with \([M+H]^+\) or \([M+2H]^2+\) as precursor ions.](image-url)
In HLM incubations, BIRB796 generates a complicated mixture of CPs. Two major mono- 
oxidized CPs (m/z 272.650, C₆H₅N₂O₢; [M+H]+ with m/z 544.283) were observed, CP544A and CP544B, for which RRTs suggest hydroxylation. For CP544A, in-source fragmentation yields an ion with m/z 263.646, consistent with an easy H₂O loss. In MS², three fragments are observed, m/z 114.093, indicating no change took place in the A-part, as well as m/z 254.129 (C₆H₅N₂O) and m/z 227.141 (C₆H₅N), consistent with AB(C+H) for AB(C+H) and AB(C+H) respectively, most likely due to a H₂O loss, indicating hydroxylation in the AB-part. For CP544B, the modification can be attributed to the A-part, because an A+CO+O fragment (m/z 216.077, C₆H₅N₂O) is observed. Another major conversion product is CP552 (m/z 251.645, C₆H₅N₂O; [M+H]+ with m/z 502.283), consistent with a combined O- and N-dealkylation (effective loss of C₆H₅) from the morpholine ring. The observation of AB(C+O), AB(C+H, A+CO), and A+CO fragments in the MS² spectrum confirms this interpretation. CP458 (m/z 229.632, C₆H₅N₂O; [M+H]+ with m/z 458.255) is another CP, resulting from a double N-dealkylation in the morpholine ring, thus involving the effective loss of C₆H₂O (dihydrofurano). This modification is supported by the observation of an unmodified AB(C+O) fragment.

A major product CP415, observed as single-charge ion (m/z 415.215, C₆H₅N₂O; effective loss of C₆H₅NO), results from O-dealkylation of the ethyl-morpholino group (loss of D). This and the related CP413 (m/z 413.199, C₆H₅N₂O; effective loss of C₆H₅NO), which is consistent with subsequent hydroquinone formation, have been observed by EC conversion [13]. Although the modification into CP415 can be quite unambiguously identified from the analysis of confirmed fragmentation and RRT results obtained from MS² data, showing fragments due to AB(C+O) and A+CO, indicating the changes are not in the AB-part, and an ion with m/z 160.078 (C₆H₅NO, 11 ppm) consistent with the C-part. Another CP (m/z 526.285, C₆H₅N₂O; m/z 351.192, C₆H₅N₂O; [M+H]+ with m/z 1051.555) is a dimeric product involving two new (C–C) bonds, as four hydrogen atoms are missing. Other MS² fragments AB(C+O) and A+CO are observed, indicating the linkages must be in part C or D. This compound shows a strong preference for the double-charge ion, both in the parent and some CPs. This is especially striking considering the relatively small differences in relevant parameters such as size, polarity or number of nitrogen atoms as compared to, for example, DMPIP. In fact, the exact position of one of the protonation sites can be deduced by following the change from double-charge to single-charge preference in the CPs (see Table 3). All CPs retaining the morpholine-N prefer the double-charge state, but as soon as the morpholine-N is replaced by a hydroxyl group (CP459) the single-charge ion becomes more abundant. Thus, the morpholine group is protonated at the N-atom.

Structure elucidation of TAK-715 conversion products
Fragmentation analysis of TAK-715 and its conversion products is not trivial. TAK-715 or CP544 shows a very complicated fragmentation tree with a large number of fragments. On top of that, the fragmentation pattern changes drastically in some CPs. This can give additional information, but restricts the usefulness of characteristic parent fragments in the profile-group approach [16,17]. In MS², TAK-715 (m/z 400.148, C₆H₅N₂O) shows two major fragments (see Figure 6 and Figure 2D). A loss of water (m/z 382.137) is probably faved by an equilibrium between the amide moiety and its hydroxyamine resonance structure, stabilized by coupling of the aromatic systems of the phenyl and the pyridine ring and an intramolecular hydrogen bond to the pyridine nitrogen (see Figure 6). The other major fragment F279 (m/z 279.095, C₆H₅N₂S) corresponds to the loss of the benzamide (C₆H₅N₂S → C=O)NH; 121.053 Da). Although fragmentation of the amide bond would be expected with complementary fragment ions with m/z 105.034 due to C₆H₅C=O and m/z 296.122 [27], the fragment ion F279 is observed instead. This unexpected fragmentation is probably also a consequence of the stabilization of the amide bond. This cleavage is also frequently found in the MS² spectra of the CPs, and can thus be used to exclude modification in this phenyl ring. Additionally, several minor fragments are observed. One is the ion F297 (m/z 297.106, C₆H₅N₂OS) which can only be explained by an unusual rearrangement of the amide oxygen from the hydroxylamine form, first leading to the structure proposed in Section 4 of the Supporting Information and then to the loss of benzo[nitrile (C₆H₅C=NO, 103.042 Da). Secondary fragmentation is considered uncommon in a single fragmentation experiment in ion-trap MS instruments. However, several fragments of TAK-715, which require two bond cleavages for formation, were observed both in MS² and MS³. The fragment F264 (m/z 264.072, C₆H₅N₂S) originates from a loss of CH₃ from F279. This is supported by observing the fragmentation pathway m/z 400.148 → m/z 279.095 → m/z 264.072 in the MS² experiments. Based solely on this fragmentation, it is unclear whether the methyl radical is lost from the methyl or the ethyl group. However, the fragment F224 (m/z 224.053, C₆H₅NS), which is observed in MS² and in MS³ via the pathway m/z 400.148 → m/z 279.095 → m/z 224.053, is helpful in this respect. The difference in elemental composition between F279 and F224 is C₆H₂N which is consistent with the loss of propionitrile from the ethylthiazole part (carbon-1, carbon-2, carbon-3 and nitrogen-7; see Figure 6). The ethyl group is thus no longer present in the F224 fragment. By observing an additional pathway m/z 400.148 (→ m/z 279.095) → m/z 224.053 → m/z 209.029, where CH₃ is lost from the F224 fragment, the ethyl group is excluded as source of this radical. The remaining two minor fragments in MS², F252 (m/z 252.084, C₆H₅NS) and F197 (m/z 197.042, C₆H₅S), result from the loss of HCN from the pyridine ring of the ions F279 and F224, respectively. Next to the analysis of the parent fragmentation, the 1H-NMR signals of TAK-715 were assigned [14]. Large-scale incubation of TAK-715 with BLM in buffer revealed that some CPs were obtained in sufficient preparation quantities to allow 1H-NMR spectroscopy. These data are shown in Table 5 and discussed here only in context of the identification by MS²; MS² spectra of most of the CPs are included in Section 8 of the Supporting Information. Three fragments were picked as profile groups, representative of different parts of TAK-715: (1) F224 and F264 representing fragments, which is due to the loss of benzylamide, was used to observe or exclude modification in the cleaved phenyl ring, (2) F264 was employed as representative of the CH₅ group (carbon-14), and (3) F224 or its corresponding fragments which is representative of the ethyl group (carbon-2 and -3). The TAK-715 CPs found with all conversion methods are summarized in detail in Table 4. CP416A (m/z 416.143, C₆H₅N₂O,S) is one of the mono-oxygenated products. In MS², it shows a fragment with m/z 277.080 (C₆H₅N₂S) which corresponds to F279 + O-H₂O. Although both an OH group and an N-oxide can show the loss of H₂O, the RRT points to hydroxylation [18]. The H₂O loss suggests the presence of an α-H, enabling a 1,2-elimination, thus an OH group in an aliphatic rather than in an aromatic system. Furthermore, an MS³ fragment (m/z 262.056, C₆H₅N₂S), corresponding to F264 O-H₂O, seems to exclude the modification of the CH₅ group at carbon-14 (Figure 6). This would lead to the conclusion that one of the ethyl carbons has been hydroxylated. However, NMR analysis [14] suggests otherwise: All aromatic protons are still present, only the m-methyl-phenyl ring protons are shifted to a lower field. The triplet and the quartet of the ethyl group are unchanged, whereas the singlet of the methyl group has shifted to a higher field. Unfortunately, as the signal is masked by a solvent signal, it cannot be integrated. Nevertheless, it still proves hydroxylation of the CH₅ group at carbon-14. This apparent mismatch between the MS² and the NMR data can be explained by assuming that the H₂O loss is accompanied by the rearrangement of a remote hydroxyl to carbon-14. This would explain the loss as well as the loss of the apparently unmodified CH₅ group as CH₅. In conclusion, the hydroxylation in CP416A was assigned to carbon-14. CP416B (m/z 416.143, C₆H₅N₂O,S) is another mono-oxygenated product. In the MS² spectrum of CP416B, an ion with m/z 295.092 (C₆H₅N₂O,S, 6 ppm), corresponding to P297 + O-H₂O, is more dominant than the ion with m/z 277.080, corresponding to P279 + O-H₂O.
A strong indication for hydroxylation of the ethyl moiety is found in a fragment with m/z 372.118 ($C_{15}H_{22}NO_2$), consistent with a loss of acetaldehyde ($C_2H_4O$); no corresponding fragment is observed for the parent. Ethyl hydroxylation is supported by NMR data: [14]. The triplet of the three protons on carbon-3 has changed to a doublet at slightly lower field and the proton at carbon-2 is still split into a quartet but the signal has shifted to a lower field by almost 2 ppm. Unfortunately, integration of this signal was not successful because of a very strong overlapping water signal at ca. 4.9 ppm. All other protons were accounted for by integration: the methyl singlet and all aromatic signals are unchanged. In conclusion, hydroxylation in CP416B has taken place at carbon-2 in the ethyl group.

CP432A (m/z 432.138, $C_{24}H_{43}N_7O_9S_2$) is a double-oxygenated product. The fragment ion in MS² with m/z 293.074 ($C_{19}H_{22}NO_2$) corresponds to $F_{279}+2xO-H_2O$. In MS³ of this fragment, a fragment ion with m/z 250.056 ($C_{17}H_{22}NO_2$), consistent with a homolytic cleavage of a C-H or, reveals a modification in the ethyl group. At first, this could suggest that both modifications took place in the ethyl group as otherwise the loss of an ethoxy radical ($C_2H_4O$) would have been expected. However, as already discussed for CP416A, H₂O loss from a hydroxylation at carbon-14 can result in hydride rearrangement from remote positions. In the fragment with m/z 293.074, the water loss from the methyl group has already occurred, probably forming a carbonyl group from the hydroxyl group in the ethyl moiety, which could explain the elimination of the $C_2H_4O$. The NMR data [14].

Figure 6: Structure of TAK-715 and proposed structures for its fragment ions in MS² and MS³. The fragmentation pathway is based on ion-trap MS² data, as outlined in the text.

Table 4: TAK-715 and its conversion products (CPs), showing elemental composition of the CP and the change (Delta) compared to the parent compound, the occurrence of some specific fragments indicated in the text, proposed identification and site of modification (refer to Figure 6), relative retention time (RRT) and the technique by which a particular CP is generated.
The MS data for CP430B (m/z 320.085, C$_{16}$H$_{14}$N$_4$O$_2$S), an isomer of TAK-715. In MS$^2$, a fragment ion with m/z 320.085 shows the loss of CO (m/z 280.091, C$_{16}$H$_{12}$N$_4$S$^+$) to give a neutral 115 Da loss to the ethyl group as the HS=C=NS loss. An additional fragment with m/z 313.136 (C$_{16}$H$_{15}$N$_3$O$^+$), consistent with a loss of HS-C=NS from the ion with m/z 372.119, links the 60.021 Da loss to the ethyl group as the H$_2$O-C=NS loss (see above). Two other major products are isomers from a dehydrogenation reaction (CP399A and CP399B; m/z 398.134, C$_{16}$H$_{14}$N$_4$O$_2$S$^+$). The modification did not occur in the benzamide part, because in MS$^3$ both CPs yield the fragment ions F$_{297}$-H$_m$ (m/z 295.089, m/z 326.097 (C$_{16}$H$_{15}$N$_3$O$^+$), consistent with a common amide bond cleavage. This results in a fragment ion with an amine group at the pyridine ring and the loss of a neutral (C$_2$H$_4$O). In MS$^3$, the fragment with m/z 308.085 shows the loss of CO (m/z 280.091, C$_{16}$H$_{15}$N$_3$S$^+$). This is a general loss of water and CO points to the presence of a carboxylic acid function. In the NMR spectrum, [14], no changes are observed in the number of aromatic protons or in the shifts for the protons in the phenyl and the pyridine ring. Furthermore, the ethyl group signals are unchanged. This indicates a carboxylic acid group in CP430B on carbon-14. Indeed, this also explains the shift to an even lower field than in CP416A of the m-methylphenyl ring protons.

CP446 (m/z 446.118, C$_{16}$H$_{14}$N$_4$O$_2$S$^+$), the fifth compound of which NMR data were obtained, contains a triple-oxygenation and a dehydrogenation. Its fragmentation pathway m/z 446.118 → m/z 324.080 → m/z 296.083 is equivalent to the pathway 430.123 → m/z 308.085 → m/z 280.091 observed in CP430B, keeping in mind the difference of one oxygen atom between the CPs. Therefore, the carboxylic acid group at carbon-14 is expected to be present in CP446 as well. Further analogies between CP446 and CP304B are observed in the aromatic region of the NMR spectra where shifts, integrals and coupling are almost identical. This suggests not only a carboxylic acid on carbon-14 but also the localization of the third additional oxygen atom in the ethyl group. Indeed, the proton signals for the protons on carbon-2 and carbon-3 are identical to those in CP416B and CP432A. To summarize, CP446 is modified to a carboxylic acid on carbon-14 and a hydroxyl moiety at carbon-2. A combination of the modifications found in CP416B and CP430B possibly leads to CP446, keeping in mind the undefined stereochemistry at carbon-2.

These five CPs were observed with both BM3 mutant M11 and HLM incubations. In HLM incubations, five other CPs were observed, two of which were also produced by BM3, however, not abundant enough in the large-scale incubation to justify purification. Therefore, their possible structures have to be derived from MS$^3$ data only.

CP432B (m/z 432.138, C$_{16}$H$_{15}$N$_3$O$^+$) is another double-oxygenated product. The MS$^3$ data suggests that both hydroxylations have taken place in the ethyl group, probably one at each carbon. The main clue is a fragment with m/z 372.119 (C$_{16}$H$_{15}$N$_3$S$^+$), consistent with a loss of 60.021 Da, due to either C$_2$H$_4$O or two consecutive losses of H$_2$O. An additional fragment with m/z 313.136 (C$_{16}$H$_{15}$N$_3$O$^+$), consistent with a loss of H$_2$O from the thiazole ring is only possible after the loss of the ethyl moiety as C$_2$H$_4$O. There are no MS$^2$ data for CP430A (m/z 430.125, C$_{16}$H$_{14}$N$_4$O$_2$S$^+$, 6 ppm) which is another double-oxygenated and dehydrogenated product. In MS$^3$, a fragment ion with m/z 308.086, observed, corresponding to the loss of water and 104 Da (C$_2$H$_4$O), similar to CP430B. The ion with m/z 308.086 yields informative MS$^3$ data. A neutral loss of CO to m/z 280.092 (C$_{16}$H$_{14}$N$_3$S$^+$), also observed in CP430B, indicates a carbonyl group. The subsequent loss of CH$_3$=CHC=NC (acrylonitrile) to an ion with m/z 227.063 (C$_{16}$H$_{14}$NS$^+$) indicates that the earlier water loss involves two protons from the ethyl group. Otherwise, a propionilure (H$_2$C=NC$^+$), as observed for the parent (F$_{297}$ > F$_{224}$; m/z 279.095 → m/z 224.053), would be expected. Thus, with an OH group at the ethyl, an aldehyde at carbon-14 is the only option to explain both the mass shift and the CO loss.

The photolytic conversion of TAK-715 produces four major and one minor CPs. One of the major products is CP400 (m/z 400.149, C$_{16}$H$_{14}$N$_3$O$^+$), an isomer of TAK-715. Remarkably, in MS$^2$ of CP400, the H$_2$O loss, which is dominant in TAK-715 and most of its CPs reported here, is no longer favoured. As a result, F$_{297}$ is now the most abundant fragment, rather than F$_{279}$. Instead of F$_{224}$ and F$_{197}$, a fragment ion with m/z 254.100 (C$_{16}$H$_{15}$N$_3$S$^+$) is observed, which is due to the HCONO loss from F$_{297}$. A structure proposal for the isomer is given in Section 4 of the Supporting Information. In fact, in MS$^3$ of this TAK-715 isomer, generated by intermolecular photodegradation in solution, was predicted as a gas-phase rearrangement product (see above). Two other major products are isomers from a dehydrogenation reaction (CP399A and CP399B; m/z 398.134, C$_{16}$H$_{14}$N$_4$O$_2$S$^+$).

show that the second hydroxylation has indeed taken place at carbon-14 as the aromatic signals are equivalent to CP416A, whereas the other hydroxylation is in the ethyl group as this modification did not influence the aromatic signals in CP416B either. Furthermore, in CP432A the doublet (3H) and the quartet (1H) for the hydroxylated ethyl group are observed at exactly the same shifts as those in CP416B. In the NMR spectrum of CP432A, integration is possible because the water signal at 4.9 ppm is far less pronounced. In conclusion, CP432A most likely contains a combination of the two hydroxylations from CP416A and CP416B although absolute stereochemistry at carbon-2 was not elucidated. The MS data for CP430B (m/z 320.085, C$_{16}$H$_{14}$N$_4$O$_2$S$^+$), which is observed next to a H$_2$O loss, probably results from a H$_2$O loss from a fragment with
C$_{17}$H$_{16}$N$_2$OS) and F$_{279}$H$_{-}$ (m/z 277.079, C$_{17}$H$_{16}$N$_3$S). From the low-abundance fragments F$_{264}$H$_{-}$ (m/z 262.055, C$_{17}$H$_{16}$N$_3$S$^-$; -6 ppm) and F$_{224}$H$_{-}$ (m/z 222.036, C$_{17}$H$_{16}$N$_3$S$^-$; -8 ppm) in the MS$^3$ spectrum of CP398B, it may be concluded that CP398B most likely results in the formation of six-member aromatic ring by bond formation between carbon-9 and carbon-20. Although the profile-group approach is less useful for the structure elucidation of TAK-715 CPs than for the other substrates, still solved cases like CP398B. The possibility to be able to propose a structure for the substrate isomer CP400 is a rare case of luck. Intriguingly, the same rearrangement as observed in solution during light incubations is, in the gas phase, key to explaining the fragment F$_{297}$ of TAK-715. The interpretation of the fragmentation of the TAK-715 CPs included a warning that gas phase chemistry can be very different from solution phase chemistry. Though water loss in solution proceeds via proton rearrangement and therefore requires an α-proton, this does not seem to be necessary in the water loss from the hydroxylated aromatic methyl group (carbon-14), a hydride rearrangement seems to be equally capable of restoring the charge neutrality. The need for an E1 mechanism due to the absence of a base in the gas phase and the extraordinary stability of water might level the playing field between proton and hydride rearrangement. The extended conjugated system might additionally enable the hydride rearrangement in the case of TAK-715 CPs.

**Occurrence of homolytic cleavages**

To a large extent, the data comply to the general finding that fragmentation of even-electron ions predominantly leads to even-electron fragment ions, which is described as the parity or even-electron rule [18,29]. However, (un)usual homolytic cleavages of even-electron ions are observed for SB203580, BIRB796 and TAK-715 as well as for several of their CPs. For SB203580, homolytic cleavage occurs exclusively in C-S bonds, as is common in methylsulfinyl or methylsulfonyl analogues [29,30]. The fragment ABC$^+$ of BIRB796 is stabilized by the formation of a semi-quinone structure, but notably is only observed with the double-charge precursor ion. The second homolytic cleavage from ABC$^+$ to AB$^+$ is then probably catalyzed by the existing radical. The methyl radical loss in TAK-715 from F$_{279}$ to F$_{264}$ and from F$_{224}$ to F$_{209}$ is more surprising. It might be speculated that this is again related to the sulfur in the aromatic system. The only expected homolytic cleavage in the CPs is the hydroxyl radical loss from the N-oxide CP394D. Although, it is clear that in DMPIP dehydrogenation induces homolytic cleavage, the uncertainty in locating the modification prevents an explanation. Amongst the TAK-715 CPs, we observe a homolytic cleavage of the hydroxylated ethyl group in CP432A. Interestingly, the hydroxylation of the methyl group does not promote its homolytic cleavage. On the contrary, CP416A still loses a methyl radical only after eliminating the additional hydroxyl group via dehydration and hydride rearrangement. Surprisingly, CP416B shows the loss of the hydroxylated ethyl group by heterolytic cleavage indicating that the methyl hydroxylation is also involved in mediating the homolytic cleavage of CP432A.

**Conclusion**

This paper reports the structure elucidation of a wide variety of related substances of four kinase inhibitors, generated in different ways. Comparison between these compound conversion tools and the effect of conversion on bioactivity is reported elsewhere [15]. This discussion may serve as an example to demonstrate the power of MS$^3$ in combination with HRMS in structure elucidation of related substances, but can evenly well be used, if one wants, to highlight the limitations of such strategy. Although the profile-group approach [16, 17] was applied as far as possible, each substrate required some adaptations in the elucidation strategy. Especially, the frequently observed deviations in the fragmentation behavior of the CPs compared to the parent compound lead to challenges in interpretation. In this respect, we may highlight the competition between H$_2$O=C=O and SO$_2$CH$_3$ losses in CPs of SB203580, the competition between CH$_3^+$ and OH$^-$ loss from an N-oxide CP394D of SB203580, the hydride rearrangement in the water loss from a hydroxylated aromatic methyl group (carbon-14) in TAK-715, the influence of the presence of the morpholino group on double-charge ion generation in BIRB796, and the intermolecular rearrangement of the fluorobenzyl group in DMPIP. It sometimes turned out to be essential to know the parent daughter relationship between the fragments which was achieved by using an ion trap instead of a quadrupole for fragmentation. The best example is found in CP410C where SO$_2$CH$_3^+$ (S-oxidation) was observed as neutral loss instead of OH$^-$ and SOCH$_3$ (unmodified sulfoxide). While known for a methylsulfinyl moiety in SB203580, the occurrence of (un)usual homolytic cleavages and radical losses in the fragmentation of the other compound classes is of interest. As such, this study does not only lead to structure elucidation of the related substances found, but also extend our understanding of fragmentation of protonated molecules in MS$^3$.

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**References**


Section 2: Additional information on BIRB796 MS³ fragmentation

Table S2: Further MS3 fragmentation of the various fragment ions of BIRB796.

<table>
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<th>Precursor in MS³</th>
<th>Fragment ions in MS³</th>
<th>Identification/Comments</th>
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<td>m/z 114.093</td>
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Section 3: Details on the structure assignment of CP305

Figure S1: (a) Structure proposals for CP305 and (b) interpretation of its fragmentation.

(a) Depicted are two structure proposals for CP305. A involves oxygenation of carbon-3, opening of the imidazole ring, and loss of methylpyridine. B has the same modifications but the atoms of the modified imidazole ring are rearranged. While A can be explained without any heavy atom rearrangements, B is more consistent with the fragmentation without gas-phase rearrangements in CID. Of both structures, a 5-ring analogue can be proposed in which the O-atom and the imine N-atom (A) or the two N-atoms are linked (B). However, the easy fragmentation makes the open variants more plausible.

Section 4: Structure proposal for TAK-715 isomer and fragmentation intermediate

Figure S2: Isomer of TAK-715 observed with Light and proposed as intermediate for TAK-715 CID fragmentation

The fragmentation of CP305 (m/z 305.077, C₁₅H₁₄FN₂O₂S⁺), is explained starting from structure proposal B. MS² results in major fragments with m/z 290.052 (C₁₄H₁₁FN₂O₂S⁺), consistent with CH₃⁻ loss, and m/z 184.044 (C₉H₁₀NO₂S⁺), consistent with an amide group at the (methylsulfinyl)benzene. MS³ on the ion with m/z 184.044 results in losses of NH₃ and HN=C=O, that is ions with m/z 167 and 141, respectively, and in loss of CH₃⁻ to an ion with m/z 169.021 (C₇H₇NO₂S⁺). MS3 on the ion with m/z 290.052 surprisingly results in fragments with m/z 140 and 123, consistent with protonated fluorobenzamide and the acylum ion due to the consecutive loss of NH₃, as well as the ion with m/z 169.021 (loss of CH₃). The observation of two (protonated) amides, m/z 184 and m/z 140, favors the structure proposal B as there is no other way to link an oxygen to both carbon-3 and -5. However, this might also be created from A in the gas phase via an intramolecular nucleophilic attack of an oxygen lone pair on the imine carbon.

For Sections 5 to 8 please refer to the supplementary material which is available on-line with the journal version and can be downloaded free-of-charge.
Chapter 4.2

EC–SPE–stripline-NMR analysis of reactive products: A feasibility study

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Abstract
Flow-through electrochemical conversion (EC) of drug-like molecules was hyphenated to miniaturized nuclear magnetic resonance spectroscopy (NMR) via on-line solid phase extraction (SPE). After EC of the prominent p38α mitogen-activated protein kinase inhibitor BIRB796 into its reactive products, the SPE step provided preconcentration of the EC products and solvent exchange for NMR analysis. The acquisition of NMR spectra of the mass limited samples was achieved in a stripline probe with a detection volume of 150 nl offering superior mass sensitivity. This hyphenated EC–SPE–stripline-NMR setup enabled the detection of the reactive products using only minute amounts of substrate. Furthermore, the integration of conversion and detection into one flow setup counteracts incorrect assessments caused by the degradation of reactive products. However, apparent interferences of the NMR magnetic field with the EC, leading to a low product yield, so far demanded relatively long signal averaging. A critical assessment of what is and what is not (yet) possible with this approach is presented, for example in terms of structure elucidation and the estimation of concentrations. Additionally, promising routes for further improvement of EC–SPE–stripline-NMR are discussed.

Introduction
Whereas hyphenation of electrochemical conversion (EC) and mass spectrometry (MS) has become an established technology, e.g., for studying drug metabolism to simulate cytochrome P450 biotransformation [1], this is not true for hyphenation of EC and nuclear magnetic resonance spectroscopy (NMR). Although EC–NMR is not an entirely new idea [2,3], it has not been extensively studied. To some extent, this may be due to the mutual influence EC and NMR conditions may have, that is disturbance of the magnetic-field homogeneity in NMR and/or of the EC processes by induction of additional currents [4]. Despite this, EC–NMR has been applied frequently and successfully to the study of processes at electrode surfaces by solid-state NMR techniques [5]. However, in the context of pharmaceutical applications, such as studying of redox pairs in vitamins [6] or structure elucidation of electrochemical products of drug-like compounds, the liquid state and bulk view is more desirable. Since the 1970s, a few groups have designed in situ EC–NMR probes where EC is carried out directly in the field region of the NMR, either in static [4] or continuous-flow approaches [2]. This allows a direct look at the EC reaction, its products and intermediates without delay. This approach has several disadvantages and limitations, such as the need for a dedicated NMR probe, the need to use the same (inert deuterated) solvent in NMR and EC, and the possible overlap of analyte peaks with signals from additives like buffers and electrolytes. Recently, EC–NMR was reported for the analysis of EC products of acetaminophen, using a conventional 60 μL NMR flow cell under steady state conditions [7]. Though high-quality spectra were achieved with commercially available equipment, the other disadvantages remain in effect. As demonstrated in hyphenation of liquid chromatography (LC) and NMR [8,9], some of these problems can be solved by the application of an on-line solid phase extraction (SPE) step to decouple EC and NMR. With respect to the application of EC–NMR in the drug discovery process, where initially only a few mg of a compound is available, a number of different setups are available [10]. Given the small sample amounts, an amperometric setup with a thin-layer cell is to be preferred over a coulometric setup. In addition, a flow-through approach is advantageous for the detection of unstable/reactive products [10,11]. Obviously, limiting the amount of sample used results in high demands on the NMR sensitivity, which is a weak spot of NMR. To enhance NMR sensitivity, there is a strong trend towards capillary or chip-based NMR techniques. While higher sample concentrations are required in capillary NMR to achieve comparable performance in terms of spectrum quality, the total amount of sample necessary can be reduced up to 100-fold [8]. In microfluidic NMR, the sensitivity is improved by decreasing the detection volume in order to achieve high concentrations for mass-limited samples, because the same number of molecules is more effectively measured in a smaller volume [12]. As EC–NMR is somewhat underdeveloped, the implementation of miniaturization and cryo technology in EC–NMR probes has not yet been reported. A relatively new trend in chip-based NMR is the stripline geometry featuring a planar resonator, which is optimised on radio frequency (rf) homogeneity, sensitivity and spectral resolution [13]. Previously, the stripline-NMR chip has been successfully used to analyse low concentrations of metabolites in human cerebrospinal fluid [14]. Moreover, the microfluidic stripline chip is perfectly suited for in situ measurements, for instance in a flow of analyte directed from the SPE to the NMR. Therefore, we chose a stripline-NMR setup for the detection of the relatively low sample amount produced by our EC setup. Though requiring low absolute amounts of sample, the stripline-NMR setup demands high analyte concentrations. To this end, the intermediate SPE step enables significant sample preconcentration next to providing a favourable solvent exchange. The complete integration into one flow system in the EC–SPE–stripline-NMR platform greatly contributes to limiting the time elapsing between generation and analysis, thus preventing degradation. In order to achieve a more realistic assessment of the current state of this EC–SPE–stripline-NMR technology, we studied EC reactions of the p38α mitogen-activated protein kinase (p38α) inhibitors BIRB796 and SB203580, which are highly relevant in contemporary drug discovery [15,16]. BIRB796 is currently studied in clinical phase III for the treatment of rheumatoid arthritis and Crohn’s disease [15]. The EC production of reactive or unstable conversion products of BIRB796 has been reported [11].

Figure 1: BIRB796 and its initial EC products. Electrochemical O-dealkylation at oxygen 9 results in the three major products shown. If not measured directly (on-line), these products undergo degradation reactions [11].
Materials and Methods

Chemicals
Acetonitrile (ACN, LC-MS grade) and formic acid (ULC-MS grade) were delivered by Bio-solve (Valkenswaard, The Netherlands). Water was purified by a Milli-Q academic from Mil-lipore (Amsterdam, The Netherlands). BIRB796 (N-[3-(tert-butyl)-1-(4-methylphenyl)-1H-pyrazol-5-yl]-N'-[4-[2-(4-morpholinyl)ethoxy]-1-naphthalenyl]-urea) (structure in Figure 1) and SB203580 (4-[4-[4-(fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine) were obtained from various sources. All other chemicals were obtained from Sigma-Aldrich (Schnelldorf, Germany).

Electrochemical conversion
EC was achieved in a ROXY EC system (Antec, Zoeterwoude, The Netherlands) consisting of a µ-PrepCell and a ROXY potentiostat. The µ-PrepCell consists of a three electrode setup with a glassy carbon working electrode, a titanium counter electrode and a HyREF™ (Pd/H2) reference electrode. A spacer thickness of 150 µm was employed resulting in a volume of 12 µL in the cell. The EC reaction mixture was supplied by a syringe pump at a flow of 5 µL/min, converted at the working electrode at a direct current voltage of 0.7 V, and directed to the SPE. The mixture consisted of 10% aqueous ACN containing 370 µM ammonium formate / 630 µM formic acid (pH 3.5) and 200 µM BIRB796. The EC of BIRB796 was monitored at 254 nm at four different flow rates of 5, 10, 20 and 40 µL/min by LC-UV (LC-20 system, Shimadzu, ‘s Hertogenbosch, the Netherlands) and analysed using a calibration curve of the standard (0–400 µM). The gradient, solvents and stationary phase used were the same as published earlier [11].

On-line Solid Phase Extraction
A SecurityGuard 4 × 2.0 mm C18 cartridge (Phenomenex, Utrecht, The Netherlands) was used. All steps were executed at room temperature at a flow rate of 5 µL/min. The trapping of the standards from the EC reaction mixture was done for 30 min, but the product mixture of BIRB796 was trapped for 60 min in order to achieve comparable concentrations for the competing quinoneimine and hydroquinone (See Figure 1). After a wash step with 99% D2O and 1% ACN-d6, the analytes were eluted with DMSO-d6 for 11 min. Finally, the wash step was repeated for re-equilibration. Additionally, a 200 µM 10% aqueous ACN solution of SB203580 containing 2% DMSO (due to solubility issues with SB203580) and 1 mM ammonium bicarbonate (titrated to pH 10 with sodium hydroxide) was trapped and eluted, but not converted, using the same protocol.

Stripline chip, probe and microfluidics
The stripline structured rf coil consists of a stack of glass or fused silica substrates with etched microfluidic channels (see Figure S1 of the electronic supplementary material, ESM) [14]. The copper structures, two ground planes and the rf coil resonator, are sputtered and etched microfluidic channels (see Figure S1 of the electronic supplementary material, ESM) into the top and bottom of the chip into the microfluidic channels to function as inlet and outlet. Via nanotight PEEK unions (P-779, Upchurch Scientific, Oak Harbor, WA, USA), both fused silica capillaries were connected to larger capillaries (150 µm ID, 360 µm OD at the inlet side and 250 µm ID, 360 µm OD at the outlet side) at the base of the magnet in order to protect the µ-PrepCell by avoiding a pressure buildup in the system higher than 10 bar. The inlet capillary was connected to the six-port switching valve (made in house) following the SPE unit. At the end of the outlet capillary, a shut-off valve (P-732, Upchurch Scientific) was used to prevent the sample from leaking out of the measurement area during measurements lasting longer than one hour. The homebuilt probe consists of an aluminum cylinder, the top of which is divided into halves in which the chip and the electronics circuit are placed [14].

Acquisition and processing
The stripline-NMR measurements were performed at room temperature in a VNMRS 600MHz Varian NMR spectrometer, operated with VNMRJ software (Agilent, St. Clara, CA, USA). The 90° pulse length was 1.95 µs at 10 W power. At the start of the experiments, the shimming of the stripline-NMR probe was once performed on a 70% ethanol solution. Then, the chip was thoroughly flushed with solvent. With the sample solution in the probe, the shims were only slightly adjusted. Tuning and matching was performed on the sample in the probe.

The elution monitoring experiments by SPE–stripline-NMR (see Figure 3) were carried out by averaging 4 scans for one data point every 15 s. All other NMR experiments were performed in stopped-flow mode. The 3 mM BIRB796 standard spectrum (Figure 4B) was measured in 256 scans with a relaxation delay of 1 s; the acquisition took 9 min. The SPE–stripline-NMR spectrum (Figure 4C) was measured by accumulating 1024 scans, taking 51 min. The EC–SPE–stripline-NMR sample (Figure 4D) was measured in an array of 25 times 256 scans with a relaxation delay of 2 s. The scans were summed afterwards. The complete experiment took 12 hr and 25 min. A total correlated spectroscopy (TOCSY) spectrum (Figure 5) was measured acquiring 128 t1 increments of 16 scans with a relaxation time of 2 s. A mixing time of 50 ms with MLEV-17 spinlock was used [17]. The experiment took approximately 2 hr and 45 min. A correlation spectroscopy (COSY) spectrum (Figure S2 in the ESM) was measured using 256 11 increments of 256 scans on the SPE trapped sample [18]. The experiment took 45 hr.

The 1 mM standard of BIRB796 depicted in Figure 4A was measured accumulating
Results and Discussion

The initial step in the EC–SPE–stripline-NMR setup was the EC of the substrate at a thin-layer electrode in the flow-through setup. Simultaneously, the products and residual substrate were trapped on the on-line SPE cartridge. After conversion/trapping, buffer salts and protonated solvents were washed away. Finally, substrate and products were eluted with DMSO-d₆ into the stripline-NMR probe. There, ¹H NMR as well as COSY and TOCSY spectra were recorded with excellent mass sensitivity.

Optimization of EC and SPE steps

The EC conditions were transferred from a method published earlier [11] which used the same EC setup but with a ReactorCell™ instead of the µ-PrepCell. It was expected that a higher concentration of substrate could be converted with the µ-PrepCell. Indeed, instead of the low to medium conversion at 10 µM with the ReactorCell™ [11], ~90% consumption of BIRB796 was observed at 200 µM and 5 µL/min with the µ-PrepCell as assessed by LC–UV. The manufacturer indicates that the optimal flow rate of the µ-PrepCell to be between 20 and 50 µL/min. As a decrease in product consumption to ca. 50, 30 and 5% was observed for 10, 20 and 40 µL/min, respectively, the original flow rate of 5 µL/min used in the ReactorCell™ was considered optimal.

Reconstructed SPE–stripline-NMR chromatograms. Elution of BIRB796 (dots) and SB203580 (boxes) from the SPE was monitored in real time by stripline-NMR using ¹H signals from a tert-butyl and a methyl group, respectively. The calculated elution time served as basis for the alignment of analyte peak and measurement area.
deviation of ~1 min leads to only 50% signal reduction, and on the sensitivity of the NMR experiment used to follow it, because multiple NMR spectra can be added to create one data point while following the elution. Both advantages are not to be underestimated when using such a highly experimental setup. However, the experimental data suggests that the signal would be strong enough to follow the elution using single scans instead of the four scans used per data point in these experiments. This would allow increasing the data point frequency from 0.07 to 0.33 Hz, thus enabling the detection of peaks with FWHM ≥12 s (7 data points above 10% signal). Thus, the elution volume at half height could in principle be reduced to 1 µL which would result in a ca. 10-fold gain in analyte concentration or equal reduction in substrate consumption through improvement of the SPE dimensions. At the moment, the substrate consumption of the EC–SPE–stripline-NMR is six times less than in the conventional NMR. When considering that only one or two percent of the sample was actually measured in the EC–SPE–stripline-NMR, this difference theoretically rises to approximately 300-fold.

In order to assess whether the SPE step has an influence on the stripline-NMR, we first measured a 3 mM standard of BIRB796 by direct infusion into the stripline-NMR probe. The results were compared to a 30 min trapping and elution of a 200 µM standard. The aromatic region of the BIRB796 spectra obtained under different experimental conditions is presented in Figure 4 whereas the corresponding chemical shifts and J-couplings are summarized in Table S1 of the ESM. All C-H signals are found at the same chemical shifts in both measurements; the maximum deviation is about 0.05 ppm for proton 35. Similar differences are observed between conventional spectra in either DMSO-d_6 or ACN-d_3 which indicate that most likely small differences in residual ACN concentration cause these effects. Additionally, the chemical shifts of BIRB796 measured by conventional NMR in DMSO-d_6 do not deviate by more than 0.05 ppm from the stripline-NMR measurements in the same solvent (see Figure 4 and Table S1 of the ESM), except for protons 12, 16 and 17 whose peaks change positions in the spectra between Figure 4A and Figure 4D. This allows comparison to off-line EC samples measured with conventional NMR. The exchangeable protons attached to the urea nitrogen atoms (N20 and N23) are no longer observed in the sample measured by SPE–stripline-NMR which indicates a very efficient exchange with deuterons. By comparing the average signal-to-noise ratios (S/N) of several peaks in the stripline-NMR and in the

![Figure 4](image)  
Figure 4: NMR spectra of the aromatic region of BIRB796. For the numbering, please refer to Figure 1. Spectra measured A) in conventional NMR (1mM, 618 scans), B) in the stripline-NMR setup (3 mM, 256 scans), C) in the SPE–stripline-NMR setup (200 µM, 1024 scans), and D) in the full EC–SPE–stripline-NMR setup (product mixture generated from 200 µM, 25x256 scans). The spectra displayed are limited to the aromatic region for better visibility. The chemical shifts and J-couplings are described in Table S1 of the ESM. The product mixture (D) is further described in the next section and Table S2 of the ESM.

![Figure 5](image)  
Figure 5: TOCSY spectrum of BIRB796 standard in the stripline-NMR
SPE–stripline-NMR spectrum, a concentration of 2.4 mM (12-fold) could be estimated for the SPE–stripline-NMR experiment which fits well to the previously calculated 15–20-fold concentration at half-height.

Furthermore, we recorded a COSY spectrum of BIRB796 by SPE–stripline-NMR. All expected coupling peaks were observed (see Figure S2 in the ESM) in effectively measuring as little as 360 pmol of sample. Unfortunately, an extensive measurement time (45 hr) proved necessary, as an attempt at recording a shorter COSY spectrum on the 3 mM standard was not successful. In the stripline-NMR experiment, relatively broad lines are found to which the long measurement time without lock might contribute. For a COSY, the diagonal peaks are in-phase whereas the cross-peaks have an anti-phase character, which may lead to vanishing signals in the case of broad peaks. This is in contrast to a TOCSY experiment, in which all peaks show in-phase absorptive lineshapes. It makes the TOCSY a more suitable experiment in our case. Therefore, we tried a TOCSY experiment on the standard, which took only 2 hr and 45 min and provided a much clearer correlation. As can be seen in Figure 5 and Figure S2 (see ESM), the same 3J-couplings between protons 11 and 12, 15 and 16, and 17 and 18 are observed in the COSY and in the TOCSY spectrum.

EC–SPE–stripline-NMR

After an initial validation of the SPE–stripline-NMR part of the integrated platform, the next step was the measurement and structural analysis of the EC products of BIRB796. For assessment of the possibilities and limitations of the platform as well as for validation of the structural analysis, the on-line spectra are compared to reference spectra obtained off-line by collection, evaporation and reconstitution with a conventional NMR probe. There is a significant difference in the overall time consumption of the experiments which stresses the advantage of the EC–SPE–stripline-NMR over the conventional approach for the detection of reactive products and is shared by the larger scale flow approach [7]. The preparation for the conventional NMR measurements often took more than a regular working day (see ESM Section 3), meaning that for between 8 and 24 hr the sample was subjected to degradation and for at least 7 hr at room temperature. In the EC–SPE–stripline-NMR, the time delay between generation and analysis was ca. 80 min. Though the NMR measurement itself was significantly longer, the products are far less likely to degrade in the elution solvent than in the reaction solvent, for example by acid-catalysis hydrolysis or dehydration [11]. Figure 6A shows that degradation is not observed in the EC–SPE–stripline-NMR setup.

For BIRB796, three main products are expected from EC–MS experiments [11]. The EC reaction is an oxidative O-dealkylation at the ether function. It produces 2-morpholinoethanol, a quinoneimine and a hydroquinone structure (Figure 1). 2-morpholinoethanol was not observed in the EC–SPE–stripline-NMR spectrum, probably because it is too polar to be trapped by reversed-phase SPE. In the EC–SPE–stripline-NMR measurement, mainly the substrate and weak signals of the two other products are found, indicating low conversion efficiency in the EC–SPE–stripline-NMR, which conflicts with the high conversion rate measured by LC–UV. This might be due to interference of the magnetic field with the electrical currents of the EC. Unfortunately, the low conversion significantly hinders the routine application of the current setup for the structure elucidation of the products. Some improvement in the product S/N, either by increased EC conversion, by an improved SPE method or by improved stripline-NMR sensitivity, is still needed before the EC–SPE–stripline-NMR can be used for product structure elucidation independently.

However, if the structure of the products is elucidated with the off-line EC experiments measured by conventional NMR, the fingerprint can be used to identify the products present in the EC–SPE–stripline-NMR spectrum (see Table S2 in the ESM). Figure 6B shows the peaks which overlap in the aromatic region after off-line EC. These assignments were supported by a COSY spectrum (data not shown) and analysis of the J-couplings of these samples. Some peaks overlap with peaks from solvent constituents in the EC reaction mixture which complicates a clear assignment. Next to the substrate, BIRB796, the hydroquinone and quinoneimine conversion products are observed. The peaks for 2-morpholinoethanol cannot be assigned with confidence, because the aliphatic region shows a lot of interferences and only minor differences with the parent chemical shifts are expected. Having the fingerprint of the hydroquinone and the quinoneimine from the off-line experiment enables us to identify some of their peaks in the EC–SPE–stripline-NMR spectrum and thereby confirm their presence. This is shown in Figure 6A which is a zoom of Figure 4D. Comparison of the data in Figure 6B and 6C shows that the peaks of the reactive/instable quinoneimine disappear after storage as is expected from earlier studies [11].

Conclusions and Perspectives

This study revealed the potential of the hyphenation of EC and miniaturized NMR in the proposed way. The EC–SPE–stripline-NMR platform allowed the acquisition of ‘H NMR and COSY as well as TOCSY proton-proton correlation spectra at substrate concentrations compatible with a high conversion rate in EC. The spectral resolution and S/N is not as high as in the off-line experiments, but the differences are subtle considering that for the EC–SPE–stripline-NMR experiments six times less substrate was consumed. Employing a test compound which shows simpler NMR spectra, more pronounced spectral differences

![Figure 6: Aromatic region of the product mixture. A) The sample measured by EC–SPE–stripline-NMR is magnified to reveal small product peaks. B) The sample is measure off-line by conventional NMR directly after re-dissolving and C) again the next day after storage at room temperature to show the stability of the different products. The substrate peaks are marked with ‘s’, the hydroquinone marked with ‘h’ and the quinoneimine with ‘q’. The quinoneimine is less stable than the hydroquinone [11]. After storage, the quinoneimine is not detected any more. This can be used additionally to distinguish the two products. An overview of the NMR signals of the two products in a numerical format can be found in Table S2 in the ESM.](image)
between substrate and product, and a higher conversion rate in EC might have lead to even higher quality NMR data, but consequently might have overestimated the usefulness of this technology at its current state of development in a drug discovery/development setting. The fact that every step of this hyphenated technology as well as the hyphenation itself can be improved significantly, either with existing technology or with easily imaginable technological improvements, promises this technology a future place in the analytical toolbox. Even despite these possibilities, the integration and miniaturization already allows the use of 10-fold lower substrate concentrations at comparable sample volumes than recently published combinations of EC with conventional flow probe NMR [7]. Additionally, both flow-based approaches limit the time delay between product generation and analysis, thus counteracting interferences by degradation.

Possible improvements of the EC–SPE–stripline-NMR platform can be readily indicated. The relatively new stripline-NMR technology still awaits integration of recent improvements in NMR systems. For example, the addition of a deuterium lock channel would allow longer measurement times without compromising the resolution, thus improving sensitivity. However, interestingly, the maximum measurement time of 12.5 hr (12800 scans) for a 1 H NMR spectrum, applied in this study, did not result in increased line width for the 9H peak compared with the 512 scan or even the single scan spectrum of the same EC–SPE–stripline-NMR sample. Measurements of a 3 mM BIRB796 standard gave a line broadening of 0.2 Hz from single scan up to 256 scans. At longer measurement times, doublets are observed in some rare cases which may be prevented by use of a lock channel. A further advancement could be achieved by integration of cryotechnology, which has led to an up to 6-fold increase in sensitivity in other miniaturized probes [21]. This might fill an existing gap in quantitative integration which requires an S/N of 150 in order to achieve less than 1% error [22]. In the EC–SPE–stripline-NMR (12800 scans), the conversion mixture gave an S/N of 75 for the doublet of proton 18 of the substrate and of only 5 for the hydroquinone. Thus, the SPE–stripline-NMR combination is only a factor of two short of achieving sufficient quality 1D spectra from 300 µL sample material at 200 µM concentration. An additional way to improve sensitivity is found in the stripline-NMR chip. The limit of detection (LOD), defined by the number of spins in 1 Hz bandwidth, is that need to resonate to achieve an S/N of 1 in a single scan [13], is with 4×1013 [spins/sqrt(Hz)] a factor of three higher than the theoretical value. This discrepancy is mainly attributed to losses due to the use of glass as a chip substrate. Therefore, other chip substrates might improve the sensitivity significantly. A fused silica chip is currently under development, and the first tests indicate that lower LOD, thus higher sensitivity, can be achieved. Another contribution to higher sensitivity may come from an improved resolution of the chip, since the line width negatively correlates with S/N. We found an FWHM of around 3 Hz, although in a previous stripline-NMR chip we have found an FWHM of less than 1 Hz [23]. Thus, improvements in line width are also anticipated for the newly designed chip. Together with the change in chip substrate, this is expected to improve S/N by at least a factor of five. The main challenge in this study was the unexpectedly low conversion rate. If the conversion rate of 60% (long collection, estimated) to 90% (short conversion, measured) of the off-line experiments would have been achieved in the EC–SPE–stripline-NMR setup, the resulting 4 to 6-fold sensitivity increase would be sufficient for acceptable product spectra.

Furthermore, improved tuning of the elution volume of the on-line SPE step to the stripline-NMR probe, bridging the 50-fold gap between measurement and elution volume, would allow increased sensitivity or decreased substrate consumption and trapping time as well. Although a second alternative would be the use of commercially available 5 µM NMR probes, these do not promise additional mass sensitivity as reported concentrations and measurements are available to those reported for the stripline-NMR here [14]. Therefore, it is likely more beneficial to bridge this gap by miniaturization of the on-line SPE step.

In conclusion, the proposed improvements provide ample opportunity to close the small sensitivity gap to achieve a productive hyphenation of EC to miniaturized NMR, provided the conversion rate in EC can be brought to the off-line level.

Acknowledgements

This work was performed partly within the framework of the Dutch Top Institute Pharma project D2-102 (Metabolic stability assessment as new tool in the Hit-to-Lead selection process and the generation of new lead compound libraries) and partly within the research programme ACTS - Process on a Chip (P2oC), financed by the Netherlands Organisation for Scientific Research (NWO). Agnieszka Kraj, Hendrik-Jan Brouwer and Jean-Pierre Chervet (Antec, Zoeterwoude, The Netherlands) are acknowledged for their support of the electrochemistry part of the project. Hans Janssen, Jian van Os and Jan van Bentum (Radboud University Nijmegen, The Netherlands) are credited for technical and organizational support with regard to set-up and ongoing development of the stripline probe. Additionally, Roald Tiggelaar, Jacob Bart and Han Gardeniers (Twente University/Mesoscale Chemical Systems, Enschede, The Netherlands) are acknowledged for their support of the stripline-NMR chip fabrication. Frans J.J. de Kanter and Andreas W. Ehlers are thanked for their input concerning the conventional NMR measurements.

References

Section 3: Off-line EC and conventional NMR experiments

BIRB796 was converted to the product mixture in 6 hr under the conditions described in 'Electrochemical conversion'. After 2 and 4 hr, the EC cell was cleaned and refilled as operation for longer than two hours resulted in significant decrease of conversion efficiency. The product mixture was dried at 30°C under a nitrogen stream. The dried sample was then stored at −20°C overnight and reconstituted in DMSO-d6, directly prior to the NMR measurements. These measurements were executed on an Advance 500 NMR spectrometer using a 5 mm CryoProbe™ (Bruker, Fallanden, Switzerland) at 500.23 MHz.

Figure S1: 400 µm stripline, showing the stripline structure (with cap and ground planes removed) on top of the probe.

Figure S2: COSY Spectrum of BIRB796 measured in the SPE–stripline-NMR setup.
Table S1: NMR data on BIRB796. Chemical shifts and J-couplings are compared between the different NMR setups. The corresponding spectra are found in Figure 4.

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* overlap with DMSO
** overlap of peaks 12, 16 and 17
For multiplets averaged values are taken

Table S2: NMR data on the two EC products of BIRB796. The chemical shift, coupling pattern and J-coupling strength are compared between the EC–SPE–stripline-NMR setup and the experiments using the conventional NMR.

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* overlap of peaks 12, 16 and 17
** multiple broad peaks in this region
*** overlap with parent compound/solvent peaks
x product does not contain these Hydrogen atoms
Chapter 5

High temperature liquid chromatography hyphenated with ESI–MS and ICP–MS detection for the structural characterization and quantification of halogen containing drug metabolites


*BioMolecular Analysis Group, Faculty of Sciences, VU University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
*QPS Netherlands B.V., Petrus Campersingel 123, 9713 AG Groningen, The Netherlands
*DSM Resolve, PO Box 18, 6160 MD Geleen, The Netherlands
In early drug discovery programs, so-called metabolic stability assessment of drug candidates (e.g., SB-203580-Iodo and MAPK inhibitor VIII) produced by bacterial cytochrome P450 BM3 mutants and human liver microsomes were identified based on high resolution MS data. Quantification was done using their normalized and elemental specific response in the ICP-MS. The importance of these kinds of quantification strategies is stressed by the observation that the difference of the position of one oxygen atom in a structure can greatly affect its response in ESI-MS and UV detection.

Introduction

In early drug discovery programs, so-called metabolic stability assessment of drug candidates, it is important to predict the clearance of a drug by cytochrome P450 (and other) enzymes in the human liver. Early in vitro metabolic stability screening provides the medicinal chemists with information on the sites in the lead structures where metabolism is most likely to occur (the so-called metabolic soft spots) [1]. The identification and quantification of metabolites requires advanced analytical technologies and the development in this area is an ongoing process [2]. Recently, we introduced a platform for the simultaneous bioactivity testing and identification of drug metabolites [3]. Depending on the properties of the drugs investigated and the bioassay applied, the platform may be adapted to include alternative detection schemes and/or detection technologies. For instance, if the target analyte of a bioassay is the compatibility with LC mobile-phase composition [4], especially with respect to the organic modifier content. High-temperature liquid chromatography (HTLC) has been demonstrated to enable compound separation at constant (low) organic modifier content. Teutenberg et al. described the fundamentals and practical issues of HTLC in detail [5,6], including the use of low organic modifier concentrations. The benefits of HTLC combined with on-line bioassays were readily demonstrated [7]. However, a constant organic modifier content is beneficial in other instances as well. Several analyte detection strategies suffer from (significant) changes in analyte response factors as a function of the organic modifier content. This may lead to under- or over-estimated concentrations of drug metabolites present in the sample. This is certainly true for attractive tools like evaporative light scattering detection (ELSD) [8,9], electrospray ionization mass spectrometry (ESI-MS) [10] and inductively coupled plasma-mass spectrometry (ICP-MS). In the latter case, a solvent gradient does not only influence the response factor but also the stability of the ICP plasma. HTLC–ICP–AES was recently reported for the analysis of food ingredients [11]. Smith et al. pioneered the integration of HTLC with ICP-MS [12] for the quantification of a glycine conjugate of bromobenzoic acid. Since its development, ICP-MS is mainly used for quantitative trace element determination. The main application area of elemental speciation is found in environmental studies. In pharmaceutical applications, the use of ICP-MS started with the profiling of inorganic impurities and nowadays is expanding to bioanalysis as well, especially involving pharmacokinetic studies of platinum based cytostatics [13,14]. More recently, ICP-MS has to a limited extent been applied in drug metabolism studies of drugs readily detectable by ICP-MS, especially drugs containing Cl and Br, as reviewed by Gammelgaard et al. [15]. Although fluorine is an even more occurring halogen in drug molecules, low-level detection of fluorine by ICP-MS is hampered by its relatively high ionization energy. The elemental specificity, the large linear range, and its ability to quantify with a species-unspecific elemental standard are features of ICP-MS that can be used complementary to ESI-MS. Cuykens et al. reported the use of ICP-MS with isotope dilution for profiling a drug in development [16] as well as the use of ICP-MS for the detection of glutathione adducts of clozapine based on S and Cl [17]. Wilson et al. reported interesting work on metabolite detection in biological fluids with ICP-MS [18–20]. In this paper, we describe the application of HTLC in the identification and quantification of drug metabolites based on ESI-HR-MS and ICP-MS. Oxidation products of two kinase inhibitors, SB-203580-Iodo and MAPK inhibitor VIII, were generated with human liver microsomes, bacterial cytochrome P450 BM3 mutants and electrochemical oxidation.

Experimental

2.1. Materials

Human liver microsomes (HLM), pooled from 50 donors were obtained from Xenotech (Lot no. 0710619) and contained 20 mg mL⁻¹ protein. Drug metabolising bacterial cytochrome P450 BM3 mutants M02 [21] and M11 V87A [22] were provided by Dr. J.N.M. Comman- deur (section Molecular Toxicology, VU University Amsterdam), Acetunitile (ACN), methanol (MeOH) both LC-MS grade and formic acid (F) ULC-MS grade were obtained from Biosolve (Valkenswaard, the Netherlands), Purified water was produced by a Milli-Q device of Millipore (Amsterdam, The Netherlands), Applichem (Lokeren, Belgium) supplied ta-nicotinamide adenine dinucleotide phosphate (NAPDH) tetrasodium salt. Isoxalopine was obtained from Arox Medchem (Groningen, The Netherlands), Clothiapine from Enzo Life Sciences (Raamsdonkveer, the Netherlands) andloxapine from Sigma–Aldrich (Zwijndrecht, the Netherlands). SB-203580-Iodo (SB-I) and p38 MAP kinase inhibitor VIII were obtained from Calbiochem (Amsterdam, the Netherlands). Monosodium dihydrogen phosphate, disodium monohydrate sodium salt, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, ammonium acetate and dimethylsulfoxide (DMSO) were purchased at Sigma-Aldrich (Schnelldorf, Germany). The structures of all analytes are provided in Supplementary Figure S1.

2.2. Methods

Metabolic incubations with the P450 BM3 mutants had a final volume of 500 μL and consisted of 500 mM MES (pH 7.4) buffer, 2.2.1. HTLC–ICP–MS
A Shimadzu LC Ion Trap–Time of Flight (IT-TOF) MS system (Shimadzu, ‘s Hertogenbosch, the Netherlands) was equipped with an HT–HPLC 200 oven from SIM GmbH (Oberhausen,
Germany. A Waters Acquity UPLC BEH C18 1.7 µm, 2.1×50 mm column was used for separation. The column was mounted inside the oven with an in house made aluminium holder for optimal heat transfer (see Figure 1 for schematic representation of the setup). A stainless steel frit (1 µm) fixed into a Valco union (DaVinci Europe, Rotterdam, The Netherlands) was used as an inline filter. The integrated solvent preheating area and post-column cooling unit consisted of 25 cm and 30 cm stainless steel tubing (internal diameter of 0.3 mm). In all HTLC experiments, the solvent preheating area was set 5°C above the starting temperature of the gradient while the post column cooling unit was set at a fixed value of 25°C.

The IT-TOF-MS was equipped with an electrospray source and operated in positive ion mode. The interface voltage was set at 4.5 kV while the source heating block and curved desolvation line (CDL) temperature were at 200°C. Drying gas pressure was set at 62 kPa and nebulising gas was applied. MS² spectra were acquired in data dependent mode with the following settings: fullspectrum MS from m/z 200–650, MS² and MS³ acquisition from m/z 80–650 with ion accumulation of 10 ms and collision energy set at 50%. The first minute of the chromatogram was diverted to waste.

A standard mixture of 100 µM isoclozapine, clozapine and loxapine in 3% DMSO, 97% H2O was analysed in isocratic mode, with eluent composition of 95% H2O, 0.2% FA and 5% ACN or 5% MeOH (solvent Aa and Am, respectively). If not stated otherwise, a flow-rate of 300 µL min⁻¹ was applied. With 5% ACN as eluent, the temperature gradient started with 0.5 min at 110°C followed by a linear increase up to 175°C in 6 min where it was kept constant for 2.5 min. With 5% MeOH as eluent, the temperature gradient started with 0.5 min at 130°C followed by a linear increase up to 175°C in 4 min where it was kept constant for 3 min. Solvent Ba and Bm were ACN, 0.2% FA and MeOH, 0.2% FA, respectively. The column was thermostated at 50°C.

The analysis of the SB-I samples was achieved using the following temperature gradient: 5 min at 110°C, then a linear increase to 180°C in 8 min where kept constant for another 5 min. The system was cooled down and after a total runtime of 20 min, it was ready for the next injection. An isocratic solvent Aa was used with a flow-rate of 600 µL min⁻¹. MAPK inhibitor VIII samples were analysed with a temperature gradient starting from 120°C for 5 min, followed by a linear increase in 5 min to 200°C where kept constant for 5 min. An isocratic solvent composition of 15% MeOH 0.2% FA was used with a flow-rate of 600 µL min⁻¹.

Thermostability of the standards was assessed by determining the peak areas of the standards in a set of experiments where isocratic and isothermal elution was performed. With increasing the temperature up to 200°C, this experiment can indicate possible on-column degradation.

**Results and discussion**

Thermal degradation of the standards was not observed under the conditions used. In the supporting information (Supplementary Figure S2) the extracted ion chromatograms of SB-I (as an example) are shown under increasing temperatures while isocratic elution was performed. The calculated peak areas had a standard deviation of less than 5%, indicating that the compound was stable throughout the analysis.

### 3.1. Solvent effect on electrospray ionization

For the three standards, isoclozapine ([C₁₈H₂₃ClN₄O₂]⁺, [M + H]⁺ with m/z 327.1371), loxapine

![Graph](image-url)
(C$_{18}$H$_{23}$ClN$_2$O, [M + H]$^+$ with m/z 328.1211) and clothiapine (C$_{18}$H$_{21}$ClIN$_3$S, [M + H]$^+$ with m/z 344.0983), a comparison was made between the peak area in ESI-MS using either a temperature or a solvent gradient. When using a temperature gradient at 5% of organic modifier, the peak areas for the standards were affected by the type of organic modifier (MeOH or ACN) to a very small extent, as can be seen in Figure 2. In the solvent gradient, the peak areas in MeOH were higher than those in ACN (24% for isoclozapine, 17% for loxapine and 16% for clothiapine), which is somewhat to be expected because ACN is the stronger solvent, making the analytes elute at a lower ACN than MeOH concentration in the gradient. The solvent effect on the ESI-MS response obviously is a bit of a disadvantage in HTLC, where only 5% of organic modifier is used. Especially loxapine and clothiapine, eluting at higher concentrations of organic modifier in the solvent gradient, show a factor of 1.9 and 2.3, decreased ESI-MS peak areas in the temperature gradient (Figure 2), respectively.

The chromatograms of using a temperature gradient and a solvent gradient with ACN can be found in the supporting information, Supplementary Figure S3. In practical applications, not only the peak area but also the signal-to-noise ratio (S/N) is important. In the total-ion chromatogram (TIC), there is not much difference in S/N for the three compounds, when comparing the solvent and the temperature gradient. In the extracted-ion chromatograms (EIC), the S/N in HTLC mode is slightly lower than the S/N of the solvent gradient. This is consistent with the difference in ESI-MS response between the two modes as described above.

3.2. Hyphenation of HTLC to ICP-MS

The detection limits of the ICP-MS for Cl$^+$ (m/z 35), Br$^-$ (m/z 79), S$^+$ (m/z 32) and I$^-$ (m/z 127) were determined by flow injection in a 5% MeOH or ACN, 0.2% FA solution. Using optimal gas flow, the detection of SO$^+$ (m/z 48; LOD 18 µM) was found to be more sensitive than that of S$^+$ (227 µM). This is due to the occurrence of a polyatomic interference of O$_3$ (m/z 31.9898) with S$^+$ (m/z 31.9721), both having m/z 32 in the ICP-MS due to its limited resolution. Isoclozapine, loxapine and clothiapine were readily separated and detected with the HTLC–ICP-MS method. The chlorine specific detection demonstrated the hyphenation of HTLC to ICP-MS, with an LOD of 128 µM. As can be seen in Figure 3, the chromatogram of m/z 35 of the HTLC–ICP-MS analysis of a 250 µM mixture of clothiapine (5), iso-clozapine (3) and loxapine (4) resulted in five peaks with an S/N ratio above 3. Peak number 1 is due to polyatomic interference of HS$^+$ + H$^+$ (m/z 35) resulting from the high percentage of DMSO in the sample, or to the breakthrough of 32Cl$. This (the unit-mass resolution ICP-MS used in this study does not allow discrimination of these two.) Peak 2 is due to an impurity in the isoclozapine standard. HR-MS data indicate an [M + H]$^+$ with m/z 355.1325 and a monochlorine isotope pattern for this impurity. The molecular formula is C$_{18}$H$_{21}$ClIN$_2$O, corresponding to additional CO in the molecule. Unfortunately, in MS/MS of the impurity, only the loss of H$_2$O is observed. Therefore, it is difficult to make a structure proposal. Peaks 3, 4, and 5 were identified based on the HR-MS data as isoclozapine, loxapine and clothiapine, respectively.

For bromine with m/z 79, the detection limit in ICP-MS was 0.5 µM and iodine could be detected down to 80 nM. For iodine, a linear calibration plot was achieved in the range of 80 nM–80 µM (y = 3.966e4 x + 2.17e3 with R$^2$ 0.9976), the experimental conditions of the HTLC part of this are described above in Section 2.2.1. The detection limits were not as low as reported by others [15], but are sufficiently low for the detection of metabolites and impurities present in the in vitro incubation samples. Moreover, the separation method differs significantly and our injection volumes are a factor of 2–10 smaller.

3.3. Profiling bromine containing MAPK inhibitor VIII products

The ESI-MS$^+$ fragmentation pattern of MAPK inhibitor VIII (M$^+$ + H)$^+$ with m/z 415.0207, C$_{18}$H$_{19}$BrCN$_2$O$^+$ in the IT-TOF was elucidated to facilitate the identification of its metabolites. Figure 4a shows the ESI-MS$^+$ spectra and Figure 4b the proposed fragmentation pathway. MAPK inhibitor VIII contains both a chlorine and a bromine atom; the characteristic isotopic pattern is observed in ESI-MS. This feature is useful for the search of structurally related metabolites in the incubation mixture by e.g., software assisted isotope pattern recognition and isotope filtering strategies [23]. In this case, we used HTLC–ICP-MS to search for all bromine containing compounds in the metabolic incubations. Subsequently, these bromine containing metabolites were identified based on their MS$^+$ spectra in the ESI-MS measurements.

Two metabolites of MAPK inhibitor VIII were found in the P450 BM3 mutant incubation samples, which were compared with HTLC–ICP-MS (Figure 5) and LC–ESI-MS. The accurate masses of these metabolites are in accordance with a hydroxylated substrate ([M + H]$^+$ with m/z 431.0156, peak 2 in Figure 5) and a quinone-type structure ([M + H]$^+$ with m/z 444.9949, peak 1 in Figure 5). The fragmentation patterns of these metabolites indicate that the oxidations took place in the toluene substructure, present in MAPK inhibitor VIII. The in vivo metabolite M$^+$ showed tentative identification from a different fragmentation behaviour in MS$^+$ compared to the parent drug. It showed a loss of H$_2$O in MS$^+$ and subsequent loss of a bromine radical in MS$^2$, and not the toluene loss observed for the parent drug. Therefore, we propose that hydroxylation occurred at the methyl group and the water loss involves a six-member ring formation between the bromo-phenyl group and the chlorine substituted benzene ring. The loss of a halogen radical from an aromatic systems is often observed in ESI-MS/MS experiments [24]. The other metabolite (peak 1) shows a mass difference of 29.9731 u, which is consistent with the loss of H$_2$O and the gain of O$_2$ (29.9742 u, thus C$_{18}$H$_{19}$BrCN$_2$O$^+$. In MS$^+$, this metabolite shows the fragment ion with m/z 296.9789, indicating the loss of the (modified) toluene ring. The most likely way to explain the observed changes in the toluene ring is by quinone formation [25]. This apparently results in an easier loss of the complete side chain (C$_{18}$H$_{21}$O$_2$) with hydrogen rearrangement rather than the stepwise loss of toluene and CO, as observed for the parent compound. The HTLC–ICP-MS chromatogram showed an additional bromine containing compound in the sample (peak 3, Figure 5), and was found to be an impurity in the analytical standard (m/z 439.0192, proposed formula C$_{18}$H$_{19}$NOClBr$^+$: 4.8 ppm error). The acquired MS$^+$ data allowed us to propose a structure for this impurity (Figure 5). The formation of a six-membered ring by adding C=C to connect the secondary substituted amine with the primary amine fits the accurate mass and matches the fragmentation spectra. Peak 4 is the substrate, MAPK inhibitor VIII with m/z 415.0207. In the HTLC–ESI-MS chromatograms, we did not observe additional metabolites or impurities missing the characteristic bromine and/or chlorine isotope pattern of MAPK inhibitor VIII.

3.4. Analysis of iodine containing SB-I and conversion products

The major fragment in MS$^+$ of SB-I (C$_{18}$H$_{19}$IN$_3$OS$^+$, [M + H]$^+$ with m/z 486.0132) in the IT-
TOF instrument is the loss of a methyl radical, resulting in the fragment with \textit{m/z} 470.9894. Performing MS\textsuperscript{3} experiments on this radical cation produces a major fragment with \textit{m/z} 423.0227 corresponding to the loss of SO. In addition, a minor fragment with \textit{m/z} 344.0843 is observed due to the loss of an iodine radical. The limited fragmentation under these conditions indicates that unambiguous metabolite identification by MS\textsuperscript{n} will not be readily possible. Incubation of SB-I with the P450 BM3 mutants M02, M11 V87A and with HLM resulted in the formation of several metabolites (see Figure 6). This figure may be used to assess the ease at which HTLC is implemented: ESI-MS\textsuperscript{n} and ICP-MS were performed on different days and in two different laboratories (Amsterdam and Groningen). In order to achieve this, the HTLC part of the system was dismantled in the first and reinstalled in the second laboratory. Nevertheless, highly comparable chromatographic results were achieved (Figure 6). MS\textsuperscript{n} spectra obtained by HTLC-ESI-MS\textsuperscript{n} analysis of the metabolites of SB-I, indicate oxidation at the sulphur, oxidation of the dehalogenated SB-I (\textit{m/z} 376.1144) and two more oxidation products (\textit{m/z} 502.0088). S-oxidation has also been reported for the fluorine substituted analogue SB-203580 [26]. Dehalogenation of the standard (\textit{m/z} 360.1181) was observed in all samples, including the controls and dilutions from a freshly prepared stock solution. Peak 1 in the ICP-MS chromatogram (Figure 6) is observed in the enzyme blank and in the t = 0 control as well, indicating a background signal of the human liver microsomal matrix and is therefore not included in Table 1. Peak 2 in the ESI-MS chromatogram with \textit{m/z} 360.1181 can be identified as the dehalogenated SB-I and is present as an impurity in all samples containing SB-I. Increased ESI-MS peak areas in the active incubations compared to control experiments indicate that dehalogenation is catalysed by the P450s. Since iodine is no longer present in the structure, this compound is not detected in the ICP-MS analysis. The presence of sulphur provides an additional feature to quantify these metabolites, although we were able to detect S\textsuperscript{+} and SO\textsuperscript{+}, these detection limits were not sufficiently low enough to be used for quantification of these low abundant metabolites. Peak 3 is a...
metabolite present in all active enzymatic incubations but the P450 BM3 mutants produce significantly more of this oxidized dehalogenated metabolite ($m/z$ 376.1136, see Table 1). The loss of a CH$_3$SO radical indicates oxidation of the sulphur. There are two possible explanations for the formation of this metabolite, either oxidation of the dehalogenated impurity or the reductive dehalogenation of the main metabolite, the sulphonated SB-I, V87A only, an additional oxidated product with $m/z$ 502.0088 was found (peak 7). Given the fact that the retention time for this product is higher than that of the parent, the compound might be an N-oxide [27]. With the formation of this specific compound, the M11 V87A BM3 mutant mimics the in vitro human metabolism the most.

### 3.5. Response comparison of ICP-MS and ESI-MS

Table 1 not only provides information on the concentrations of metabolites, based on the response in ICP-MS, but also enables a comparison of the response factor in ESI-MS and ICP-MS. For example, the metabolite peaks 6 and 7 have the same chemical formula and the oxygen incorporated by metabolism results in only a relatively small difference in the chemical structure. However, whereas the concentrations of these metabolites are very similar (0.75 µM peak 6 and 0.72 µM for peak 7), the response in ESI-MS differs by a factor of $\sim 25$. (25.5×10$^6$ for peak 6 and only 1.0×10$^6$ for peak 7). The ICP-MS is a normalized detector for specific elements and therefore not sensitive to the difference in structure of these two metabolites. Additionally, the results of comparing the same calibration samples measured on both instruments show the difference in linearity, especially with the higher concentrations of SB-I measured from 0.80 µM of SB-I. The LOD for SB-I with HTLC–ICP-MS was 80 nM while with ESI-MS the LOD was as low as 10 nM. The linear range of the ICP-MS was found to be from 80 nM up to 80 µM, while in HTLC–ESI-MS linearity was observed from 20 nM to 10 µM. This demonstrates the potential of over- and/or underestimating the concentration of a metabolite based on its response in a regular LC–ESI-MS run. In this case, the small structural changes in the molecule and the limited linear response in ESI-MS require complementary normalized detection methods to provide an accurate assessment of the concentrations present in the sample.

Next to incubations with enzymes, we apply electrochemical oxidation to generate oxidation products of drugs. In some cases, several human relevant metabolites can be produced in this way [28]. This is a relatively clean method to produce oxidation products and therefore may support metabolite identification studies. We applied electrochemical oxidation to the iodine containing SB-I and use this additional sample to illustrate the strength of hyphenating HTLC to ICP-MS. The electrochemical oxidation products were analysed in both ICP-MS and ESI-MS configuration. The total compound concentration in this sample was 5–10 times lower than in the enzymatic incubations since the optimum electrochemical conversion conditions were found to include a substrate concentration of only 10 µM (data will be published elsewhere). Figure 7 shows the ICP-MS trace of iodine and the UV chromatogram at 254 nm. Unfortunately, in ESI-MS only the substrate was found, indicating that the concentrations of the formed products were below their ESI-MS detection limits. The latter was verified using a conventional solvent gradient analysis. Nevertheless, the concentrations of the products could be determined by the HTLC–ICP-MS analysis (Table 2). The electrochemical oxidation process converted more than 60% of the initial SB-I into six iodine containing products. The major product was formed at a concentration of 4 µM,
while other products were formed in concentrations of 1 µM or lower. In this particular case, the total concentration of all products and the parent drug was found to be the same as the total concentration of the parent drug before oxidation. This means that no dehalogenation occurred in the electrochemical oxidation and that all products detected contained iodine. If this would be the case with in vitro metabolism studies, mass balancing can be done which significantly contributes to the elucidation of the drug metabolism and to the metabolic and pharmacokinetic profiling. Moreover, the data in Table 2 and Figure 7 also indicate that not only the ESI-MS response but also the molar extinction coefficient cannot be made since it would significantly underestimate the concentrations of conversion products have the same UV extinction coefficient may significantly change upon oxidation. For instance, peaks 4 and 5 show similar response in ICP-MS, whereas their response in UV is significantly different. Therefore, in this specific case, the assumption that conversion products have the same UV extinction coefficient cannot be made since it would significantly underestimate the concentrations of products formed by oxidation.

Table 2: Retention time, peak area and determined concentration by ICP-MS of electrochemical oxidation products of SB-I from Figure 7.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Peak retention time (min)</th>
<th>Area (counts)</th>
<th>Concentration µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>2311</td>
<td>&lt;0.08 (0.003)</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>17516</td>
<td>0.39</td>
</tr>
<tr>
<td>3</td>
<td>1.8</td>
<td>9983</td>
<td>0.20</td>
</tr>
<tr>
<td>4</td>
<td>5.1</td>
<td>160583</td>
<td>4.00</td>
</tr>
<tr>
<td>5</td>
<td>7.7 (SB-I)</td>
<td>151373</td>
<td>3.76</td>
</tr>
<tr>
<td>6</td>
<td>7.9</td>
<td>29748</td>
<td>0.70</td>
</tr>
<tr>
<td>7</td>
<td>11.7</td>
<td>44491</td>
<td>1.07</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>415975</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Figure 7: Overlay of HTLC-ICP-MS (solid upper trace) and HTLC-UV (dashed lower trace) chromatograms of the electrochemical oxidation products of SB-I.

Conclusions

The combined use of temperature gradients and isocratic solvent compositions enabled the profiling and quantification of several oxidation products of SB-I and MAPK inhibitor VIII by means of ICP-MS. Information on structures of the metabolites was obtained by ESI-HR-MS experiments. The detection of drug metabolites by normalized and selective detection of a halogen using ICP-MS provides valuable and complementary information on the analytes. Whenever the halogen remains incorporated within the metabolites, they are detected, can be quantified, and even mass balancing is possible. However, if oxidative dehalogenation occurs, mass balancing approaches become more difficult, especially when more than one metabolite loses the halogen from its structure. Nevertheless, the hyphenation of HTLC to structure independent detection methods such as ICP-MS and ELSD can contribute to the toolbox of researchers for the quantification of drug metabolites and other drug related molecules, e.g., process impurities, degradation products. The importance of these kinds of quantification strategies is stressed by the observation that the difference of the position of one oxygen atom in a structure can greatly affect its response in ESI-MS and UV detection. Whether the incorporation of this atom is by P450 metabolism, electrochemical oxidation, photodegradation or any other process is irrelevant for the strategy presented in this paper.

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References


Supplementary Material

Figure S1: Structures of the compounds used in this study

Isoclozapine

Chemical Formula: C18H19ClN4

Loxapine

Chemical Formula: C18H18ClN3S

Clothiapine

Chemical Formula: C18H18ClN3O

SB-203580 -Iodo

Chemical Formula: C20H16BrClN2O

P38 MAPK Inhibitor VIII

Chemical Formula: C21H16IN3OS

Figure S2: Temperature Stability of SB-203580 -Iodo

Figure S3: Comparison of HTLC-ESI-MS and LC-ESI-MS

A: Temperature gradient with 5 % ACN

B: ACN gradient isothermal 50 °C
Section 6

Summary

Chapter 6.1

Conclusions and perspectives
This thesis aims at exploring new applications of high-resolution screening (HRS). Therefore, our goal was to improve the corresponding platforms and extend their possibilities, mainly by further integration of chromatography, mass spectrometry, bioassays and especially innovative synthetic methods. We developed HRS platforms for two targets, p38α mitogen-activated protein kinase (p38α) and soluble epoxide hydrolase (sEH), which were previously not accessible by HRS [Chapter 2]. Furthermore, by integrating four complementary modification methods with the p38α HRS platform, we achieved an efficient and integrated approach to generate and screen metabolite-like lead libraries, yielding structure-affinity relationships [Chapter 3]. We thoroughly analyzed the possibilities and limitations of structure elucidation of related substances by liquid chromatography–(on trap–time-of-flight) high-resolution mass spectrometry (LC–HR-MS) within the framework of HRS on basis of the kinase inhibitor derived metabolite-like lead libraries [Chapter 4.1]. Finally, we peaked into the future of HRS which may feature miniaturized NMR for additional structure elucidation [Chapter 4.2] and/or inductively coupled plasma MS (ICP-MS) for absolute quantitation [Chapter 5] integrated directly into the HRS platform.

As far as the development of HRS bioassays is concerned, on the example of p38α, we demonstrated the first on-line post-column bioaffinity assay reported for any enzyme target and integrated it into an HRS platform [Chapter 2.2]. For the sEH HRS platform, we were mainly interested in making the possibilities of HRS for metabolism studies available to this novel target and therefore took a very classic approach [Chapter 2.3].

For the p38α HRS platform, we additionally introduced new solutions for the screening of highly lipophilic compounds. The use of fused silica capillaries demonstrates that there are still possibilities to improve upon current HRS bioassays technology, even in aspects that have been as extensively studied as post-column reactors, in this example, the employment of chromatographic equipment, which was shown to achieve excellent performance of the p38α and the sEH HRS platforms. For the p38α HRS platform, this is indicated by excellent figures-of-merit, such as a 2-factor of ca. 0.8 and an S/N of up to 100, and an affinity ranking in accordance with literature values. Unfortunately, the only tested compound of which affinity data on non-phosphorylated p38α was available, was unconvincing binding kinetic, which could not have been predicted by a quantitative comparison of the IC_{50} values for validation purposes impossible, due to the limited incubation times achievable in HRS. Nonetheless, its affinity was detected as were those of representative compounds of all three types of ATP-competitive binding.

The sEH HRS platform featured an S/N>60, though quality would have certainly profited from the use of state-of-the-art equipment. We could very reliably match affinity and small-molecule structure and even tackle isobaric and many isomeric compounds. This holds for both HRS platforms, but was more extensively demonstrated for p38α [Chapter 2.2, Chapter 3] than for sEH [Chapter 2.3]. The clear advantages of HRS over HTS with respect to the bioassay level are exemplified by a decreased p38α concentration and a similar sEH concentration at significantly reduced incubation time. Obviously, the ability to analyze mixtures is an important advantage of HRS over HTS as well.

For both HRS platforms, we successfully tackled issues with enzyme stability. In addition, for the sEH HRS platform, we overcame issues with low substrate solubility. Furthermore, during the development and the HRS campaigns [Chapter 2, Chapter 3], we observed a number of interferences which are clearly visualized by the HRS bioassays. Examples are the auto-fluorescence and peak tailing in the p38α HRS platform, which interfere with the IC_{50} measurements [Chapter 2.2, Chapter 3.2], and to some extend the precipitation issues during the development of the sEH HRS bioassay [Chapter 2.3]. While the visualization of interferences might be seen as a disadvantage, we believe that it in fact is infinitely preferable to spend efforts and resources to address these issues as soon as confidence levels are low. Therefore, we regard the ability of the HRS platform to resolve interferences as one of the major strengths of this approach.

There are several interesting directions in which both the p38α and the sEH HRS platform may be further developed. Parallel bioassays (two or even multiple), similar to the one developed for the estrogen receptors α and β [1], for binding selectivity analysis should be mentioned in this context. These could be applied to investigate selectivity over close family members or generally problematic off-targets. For example, in the case of p38α, the closely related kinases JNK-1, JNK-2 and JNK-3 could be simultaneously monitored for selectivity during lead optimization [2]. Additionally, detection of selectivity for the different p38 isoforms might lead to molecular probes which could be used to investigate the biological role of p38β, γ and δ in more detail [3]. Concerning off-targets, the increasing interest in the novel concept of translational research may lead to the exploitation of the large body of knowledge on p38 inhibitors in future drug discovery campaigns. For example, the suspected off-target interactions, related to certain side effects observed in the clinic, might be included in selectivity studies.

In any case, next to selectivity detection, future developments of HRS bioassays lie in miniaturization, because the reduction in both reagent consumption and sample volume has important advantages [4]. Using less target protein helps to control the cost of HRS and thus improves its competitive position versus HTS. Enhanced mass sensitivity, possibly in combination with pre-concentration through the integration of on-line SPE, might finally allow HRS analysis of clinical samples in the future.

We further advanced integration in HRS platforms, synthesizing metabolite-like lead libraries and analyzing them for structure and affinity in one platform or even in one hyphenated system. Combining synthesis and analysis in one workflow resulted in an efficient exploration of neglected chemical space around a substrate scaffold, consequently yielding structure-activity relationships with the input of no more than the substrates. These substrates were important p38α inhibitors, including a lead (DMP170), a widely used molecular probe (SB203580) and clinical candidates (BIRB796 and TAK-715). As we focused on metabolite-like lead libraries, the need to obtain information on mechanism of action became especially apparent. The use of biocatalysts to generate metabolite-like lead libraries proved very successful early on in drug discovery. The electrochemical conversion (EC) HRS platform was certainly the most interesting integrated synthesis approach from an analytical point of view [Chapter 3.1]. The EC setup employed in this study (Chapter 3.2) showed unconvincing binding kinetic, which was seen to allow a quantitative comparison of the IC_{50} values for validation purposes impossible, due to the limited incubation times achievable in HRS. Nonetheless, its affinity was detected as were those of representative compounds of all three types of ATP-competitive binding.

The use of biocatalysts to generate metabolite-like lead libraries also offers the possibility for a new synthetic approach. A pre-column incubation step with a mixture of nucleophiles could be integrated into the EC–HRS platform, allowing the production of extensive lead libraries with a broad chemical diversity in a rapid and automated fashion as well as the direct analysis of the reaction mixtures in the same system. Especially interesting might be the integration of a second electrochemical (detection) step between column and MS which would serve to probe the redox stability of the new compounds. Through this approach, it might be possible to find leads with a reduced tendency to form reactive products [5]. This is especially interesting, if adverse effects caused by reactive metabolites are known or anticipated. The general approach of EC–HRS might also be interesting in stages of drug discovery other than lead optimization. When fitting an EC–HRS platform with a bioassay for a notorious off-target, such as HERG [6], it could be very useful as a rapid metabolic alert screening. Though EC is not a complete mimic of human metabolism, redox weak spots and the toxicological consequences of the associated modifications may be identified early on in drug discovery.

The use of biocatalysts to generate metabolite-like lead libraries proved very successful as well. The library of Cytochrome P450 BM3 (BM3) mutants applied produced a number of conversion products. The products showed increased hydrophilicity and one of them even retained full p38α affinity [Chapter 3.2]. Because large parts of the BM3 mutant library were “humanized”, a significant overlap with products from HLM incubation was found. The
fact that, through up-scaling and purification, IC_{50} determination and NMR measurements were enabled, greatly enhanced the detail of information obtained. In addition, we thereby demonstrated how the initial results of the HRS platform can be used to guide further structural and pharmacological investigation of promising conversion products.

Subsequently, we compared the EC and the biocatalytic approach to chemically (H_{2}O) and photochemically (visible light) induced transformations [Chapter 3.3]. H_{2}O was very good in making many different, more hydrophilic products, e.g., numerous oxygenated isomers. Photochemical conversion showed some unique products and can induce isomerism in the core structure, which may result in novel pharmacophores. One consequence of that isomerism is a more limited success in structure elucidation by MS [Chapter 4.1].

In comparison, all methods have their specific profile of strength and weaknesses. For example, biocatalysis and H_{2}O are most successful in increasing hydrophilicity. While biocatalysis is the most laborious method, but can also be the most selective, H_{2}O is very straightforward but often produces the most (isomeric) products. Photochemistry and EC do not favor increased hydrophilicity in the products, but photochemistry yields the most unique products. However, the EC–HRS platform is very favorable for the analysis of reactive products, due to its direct analysis capabilities.

In conclusion, all conversion methods are highly complementary and thus together present an interesting toolbox for the generation of metabolite-like lead libraries. We could show the impressive value of integrating synthesis of mixtures into the HRS strategy. The p38δ HRS platform rapidly delivered an initial structure-activity relationship for the lead libraries and can be considered as a solid starting point for further pharmacological and toxicological investigation.

Next to producing lead libraries with favorable properties, perhaps unsurprisingly, especially the biocatalytic and the chemical approach showed potential for the synthesis of metabolite standards. The novelty is that the implementation of the HRS platform allows a focus on active metabolites for further evaluation while at the same time still providing a comprehensive overview. Again, this might also be an interesting application for off-target HRS bioassays.

However, both the biocatalytic and the chemical approach will require different strategies for metabolite standard production. By mutation, a biocatalyst can be generated which exclusively or predominantly produces one metabolite that is then easily purified. The strategy for H_{2}O will rely on simultaneous purification of all (major) products, as conversion is undirected (high number of isomers) and there is little opportunity to tune the product profile. However, this will also provide standards which would be ignored in a focused approach, but might still be useful, for example when metabolites are detected in human that have not been predicted in in vitro or animal models.

Being an integral part of the HRS platforms, we closely investigated the possibilities and challenges of structure elucidation by LC–HR–MS via the special circumstances of a (HRS) screening paradigm [Chapter 4.1]. The resulting restrictions include the use of "generic" settings for the ionization and fragmentation experiments as well as prohibiting the use of elaborate MS experiments like stable isotope labeling and H/D-exchange experiments. Nonetheless, the attribution of modifications to specific parts of the molecule is often successful and in special cases allows absolute structure elucidation, for example for CP472, a conversion product of DMPIP in the EC experiments [Chapter 3.1]. The sometimes drastic changes in fragmentation initiated by comparatively minor modifications and unexpected intra-molecular rearrangements presented challenges in the LC–HR–MS structure elucidation. These rearrangements were also interesting examples of gas-phase chemistry, such as the electrocyclic rearrangement in the fragmentation of TAK-715 (ion C1 in Figure 4.6) or the conversion of the para-fluoro-benzyl group in CP416A (derived from TAK-715) and the rearrangement of the whole para-fluoro-benzyl group in DMPIP. Additionally, we detected an unexpectedly high number of homolysolic cleavages in violation of the even-electron rule. In the process, we showed, using ion-trap fragmentation in combination with HR-MS readout by time-of-flight, the value of clearly assigning precursor-product ion relationships for structure elucidation [Chapter 4.1].

Next to the LC–HR–MS structure elucidation, we pursued an intriguing possibility to incorporate NMR structure elucidation into an HRS platform in the future [Chapter 4.2]. As starting points, we took the reactive products of BIRB796 discovered in the EC–HRS platform [Chapter 3.1]. We set out to elucidate their structure by NMR. The logical choice for an approach that can be integrated with HRS was a miniaturized flow probe NMR in order to match the small sample volume and the flow-through EC system.

In the end, we combined an up-scaled electrode, an SPE step for pre-concentration and solvent exchange, and a stripline-NMR chip probe equipped to a 600 MHz NMR instrument. The system was well suited for the detection of the BIRB796 standard, and the solvent exchange was efficient enough to be able to compare the EC–SPE–stripline-NMR results to conventional (2D–)H-NMR. Unfortunately, the conversion rate of BIRB796 to its two reactive products was significantly lower than in the off-line experiments. Therefore, identification of the products could only be achieved by comparison with the conventional "H-NMR spectra, instead of by the desired independent structure elucidation. However, the platform showed promising results and there are numerous possibilities to close the existing sensitivity gap in order to allow direct integration into the HRS platform. Integrated LC–MS/NMR platforms are already used in pharmaceutical and other applications [7,8]. As combined MS and NMR structure elucidation is crucial in HRS applications as well [1], HRS platforms featuring this combined structure elucidation power are certainly a worthwhile aim.

A major enhancement of the possibilities and a major step towards a wider applicability of HRS is the integration of absolute quantitation into HRS platforms. For example, this may enable a quantitative structure-activity relationship (QSAR) directly from mixtures generated by the described modification methods. A simple injection of a dilution series of the mixture might suffice in order to obtain IC_{50} curves. Mainly due to the lack of standards, detection techniques focused on atoms rather than on molecules will be the methods of choice. Comparing with unknown compounds. Detection techniques like UV/VIS, or NMR, which focus on molecular properties, show a large variation in response, depending on the molecular structure. While the response also shows large variation between different atoms when atomic properties are detected, it is relatively easy to find a common atom in many different organic molecules.

One atom property based method, which was already discussed, is NMR. However, NMR is influenced by the chemical environment of the detected atom which has consequences for the response. Fortunately, these are much less pronounced than in molecular property based detection techniques and can be minimized by careful consideration of experimental parameters such as relaxation time. The real issue with NMR is its currently inadequate sensitivity. Yet, if this hurdle can be overcome, NMR promises quantitation and additional structure information at the same time.

ICP-MS is much more sensitive and its response is virtually independent of the chemical environment, due to the constant atomic ionization efficiency and the huge difference between molecular bond energy and the atomic ionization energy [9]. Unfortunately, the main elements of organic molecules, hydrogen, carbon, oxygen, and nitrogen, cannot be targeted by LC–ICP–MS for various reasons. However, many pharmaceuticals contain a halogen and/or sulfur atom and there are even examples containing metal atoms. While we measured bromine and iodine very sensitively by ICP–MS, this was not the case for sulfur and chlorine [Chapter 5]. The observed isobaric polyatomic interferences may be resolved using reaction/collision cells or an ICP-HR-MS instrument [8]. This might allow sensitive quantification of sulfur or chlorine containing molecules, where detection limits with unit mass resolution ICP–MS were a factor of 30 or 200, respectively, short of HRS compatible quantitation [Chapter 5]. ICP–MS relies on stable solvent conditions and is therefore not compatible with classic solvent gradients [10]. High temperature LC (HTLC) presents an
elegant way to achieve efficient separation at isotropic solvent conditions [Chapter 5]. HTLC is not only interesting for ICP-MS compatibility, but may also present a solution to reduce the organic modifier influence on post-column bioassays. Though the ESI-MS response is decreased at low organic modifier concentrations, the high sensitivity of ESI-MS usually allows a favorable compromise. In its application to drug discovery and related fields, we have demonstrated many strengths and possibilities of HRS and encountered some of its limitations. However, the role HRS platforms will play in the future depends on many factors. At the moment, the development status of HRS platforms limits their application to niches where separation of complex mixtures is a key element, for example screening of natural product libraries [11,12]. The specific niche will determine the HRS platform’s outward appearance, e.g., whether pre-column affinity selection or post-column bioassays are employed, or whether on-line or at-line hyphenation is favored [Chapter 1.1.2]. Miniaturization may present a way to reduce HRS cost [4] and thus enhance its commercial competitive position versus HTS. However, knowledge presents a much greater hurdle towards the implementation of HRS platforms. Currently, a user has to be trained in all analytical and pharmacological disciplines related to the HRS platform. This will only change, if commercial solutions for robust and fully integrated systems as well as automated data analysis tools become available in the future. For HRS to be recognized as a real alternative in pharmaceutical screening and to create a demand for these solutions in the first place, next to maturity and user friendliness, one hurdle is potentially all-important. HRS does not smoothly fit the high-throughput batch approach dominating contemporary pharmaceutical industry and is thus very rarely seriously considered. However, this may very well change in the not so far future. The pharmaceutical industry is under great pressure to critically evaluate their R&D strategies and present more viable alternatives [13,14]. More flexible, quality-focused drug discovery campaigns and an increasing interest in flow chemistry as alternative to batch approaches may make HRS platforms an attractive tool. Flow chemistry might be integrated as demonstrated in the EC–HRS platform [Chapter 3.1] or with an intermediate on-line SPE step. With appropriate flow splitting and at substantially decreased cost, HRS might also be useful beyond discovery, for example for monitoring up-scaling or even for quality control of active pharmaceutical ingredient production based on the (side) effect rather than molecular composition. Thus, the “ideal” HRS platform of the future consists of a (multiple) modification method(s), state-of-the-art (multidimensional) separation, multiple bioassays for selectivity analysis, two independent structure analysis techniques like ESI-HR-MS (including MS/MS or MS<sup>2</sup> capabilities) and NMR, and two independent quantitation techniques such as ICP-MS and NMR, operates at micro- or even (partly) nano-scale, and can be used by scientists with diverse backgrounds. Applications might include fully automated quantitative structure-activity relationship studies and, without a modification method, the quantitative elucidation of the pharmacokinetic contributions of all pharmacologically or toxicologically relevant metabolites for metabolic profiling.

References

Chapter 6.2

Nederlandse samenvatting
Hoge-resolutie screening voor metaboliet-gerelateerde lead-bibliotheken

In dit proefschrift worden nieuwe applicaties van hoge-resolutie screening (HRS) onderzocht. Daarvoor streefden wij naar verbetering van bestaande systemen en verbreding van hun mogelijkheden. Hierbij hoorde geavanceerde integratie van chromatografie, massaspectrometrie, bioassays en in het bijzonder innovatieve synthetische methodes. Wij ontwikkelden nieuwe HRS systemen voor twee doelproteïnen, p38α mitogen-geactiveerd proteïne kinase (p38α) en soluble epoxide hydrolase (SEH) [Hoofdstuk 2]. Verder bereikten wij een efficiënte en geïntegreerde aanpak voor het genereren en screenen van metabo- liet-gerelateerde lead-bibliotheken, door integratie van vier complementaire modificatiemetho- thodes met het p38α HRS systeem, welke uiteindelijk resulteerden in structuur-affinitheids- relaties [Hoofdstuk 3]. Wij analyseerden grondig de mogelijkheden en beperkingen van structuuropheldering van gerelateerde stoffen met behulp van vloeistofchromatografie–ion-trap–time-of-flight hoge-resolutie massaspectrometrie (LC–HR–MS®) in relatie met HRS gebaseerd op de metaboliet-gerelateerde lead-bibliotheken van de kinase remmers [Chapter 4.1]. Uiteindelijk waagden wij een poging om de (mogelijke) toekomst van HRS te exploreren. Deze zou mogelijk bestaan in integreren in het HRS systeem van gemoni- aturiseerde kernspinsresonantie spectroscopie (NMR) voor verdere structuuropheldering [Hoofdstuk 4.2] en/of inductief-gekoppelde plasma MS (ICP-MS) voor absolute kwantificatie [Hoofdstuk 5].

Het p38α systeem is het eerste on-line post-column bioaffiniteitssay systeem voor een enzym, een belangrijke stap in de ontwikkeling van HRS bioassays [Hoofdstuk 2.2]. Bij het SEH HRS systeem waren wij voornamelijk geïnteresseerd in het toegankelijk maken van de mogelijkheden van HRS in metabolismeudies voor dit doelenuit en hebben we daarvoor een klassieke benadering gekozen [Hoofdstuk 2.3].

In het p38α systeem hebben we bovendien nieuwe oplossingen voor het screenen van zeer lipofiele stoffen geïntroduceerd. Het gebruik van fused-silica capillairen laat zien dat er nog steeds mogelijkheden zijn om de HRS bioassay technologie te verbeteren, zelfs in zo veelvuldig bestudeerde aspecten als post-kolom reactoren. In dit geval resulteerde de betere piekform in een betere chromatografische resolutie en nauwkeurigere verdunningsberekeningen. Wij berekenden uitstekende prestaties van het SEH systeem, welke uiteindelijk resulteerden in structuur-affinitheidsrelaties [Hoofdstuk 3]. Wij analyseren de mogelijkheden van HRS voor het maken van informatie over het structuur van een enzym, een belangrijke stap in de ontwikkeling van HRS bioassays [Chapter 4.2].

Deze zou mogelijk bestaan in integreren in het HRS systeem van gemoni-aturiseerde kernspinsresonantie spectroscopie (NMR) voor verdere structuuropheldering [Hoofdstuk 4.2] en/of inductief-gekoppelde plasma MS (ICP-MS) voor absolute kwantificatie [Hoofdstuk 5].

interessante verzameling vormen voor het genereren van metabool-gerateerde lead-bibliotheken. Zo waren wij in staat om de meerwaarde van de integratie van de synthese van mengsels met het HRS strategie te demonstreren. Het p38α systeem leverde in korte tijd een intens structuur-affiniteitsrelatie voor de lead-bibliotheken op en kan als een solide startpunt voor verder farmacologisch en toxicologisch onderzoek gezien worden. Naast het produceren van lead-bibliotheken met gunstige eigenschappen hebben, mischien niet onverwacht, vooral de biokatalytische en de chemische aanpak potentieel voor de synthese van metabolitiestaandaarden getoond. De innovatie ligt hierbij vooral in de mogelijkheid om zich voor gedetailleerd onderzoek op actieve metabolieten te focussen en toch tegelijkertijd een breder overzicht te verwerven.

Omdat het een integraal bestanddeel van het HRS systeem is, hebben wij in meer detail naar mogelijkheden en beperkingen van structuuropheldering met behulp van LC–HR-MS+ gekeken, zoals die in de bijzondere omstandigheden van een (HRS) screeningsomgeving optreden [Hoofdstuk 4.1]. De bijhorende beperkingen omvatten het gebruik van ‘gemeenschappelijke’ parameters voor ionisatie- en fragmentatie-experimenten en verhinderen van ingewikkelde MS experimenten zoals stabiële-isotooplabeling en H/D-uitwisseling. Nettomin was het relatoren van modificatieaspecten naar specifieke delen van de moleculen vaak succesvol en kon in speciale gevallen zelfs de absolute structuur opgehelderd worden, bijvoorbeeld voor CP472, een omzettingsproduct van DMPIP in de EC experimenten [Hoofdstuk 3.1]. De soms drastische veranderingen in fragmentatie als gevolg van relatief kleine modificaties en onverwachte intra-moleculaire omleggingen waren uitzonderingen in de LC–HR-MS+ structuuropheldering. Deze omleggingen waren tegelijkertijd interessante voorbeelden van gasfase-chemie, bijvoorbeeld de elektrocyclische omlegging in de fragmentatie van TAK-715, de omlegging voorgaande aan de vorming van CP305 (of zijn fragmentatie) uit SB203580, de hydride-omlegging vanuit de aromatische hydroxymethyl groep in CP416A (afkomstig van TAK-715), en de omlegging van de hele para-fluoro-benzyl groep in DMPIP. Bovendien detecteerden wij een onverwacht groot aantal homolytische breukreacties welke in strijd zijn met de even-elektron regel. Daarbij hebben wij, door gebruik te maken van de ion-trap fragmentatie in combinatie met time-of-flight HR-MS detectie, het belang van een heldere relatie tussen precursor ion en product ionen voor structuuropheldering aangetoond [Hoofdstuk 4.1].

Naast de HR-MS+ structuuropheldering verkenden wij een veelbelovende mogelijkheid om in de toekomst structuuropheldering met behulp van NMR te integreren met het HRS systeem [Hoofdstuk 4.2]. Hierbij stonden de reactieve producten van BIRB796 centraal die in het EC–HRS systeem ontdekken werden [Hoofdstuk 3.1]. Hun structuur wilden wij met behulp van NMR ophelderen. De logische aanpak gericht op integratie met HRS zou een miniaturiseerde flow-sonde NMR kunnen zijn, waarmee de kleine monstervolumes en een doorstroom-EC-apparaat goed te combineren zijn. Uiteindelijk gebruikten wij een vergrote werkelektrode en een vastefase extractie (SPE) stap ten behoeve van voorconcentratie en oplosmiddeluitwisseling in combinatie met een stripline-NMR chip sonde en een 600 MHz NMR instrument. Het systeem was goed geschikt om de BIRB796 standaard te detecteren. De oplosmiddeluitwisseling was efficiënt genoeg om een vergelijking van EC–SPE–stripline-NMR resultaten en conventioneel (2D–)1H-NMR mogelijk te maken. Helaas was de omzettingsgraad van BIRB796 naar zijn twee reactieve producten in de on-line experimenten duidelijk lager dan in de off-line experimenten. Daardoor was productidentificatie alleen mogelijk door het vergelijken met de conventionele 1H-NMR spectra in plaats van door de bedoelde onafhankelijke structuuropheldering. Toch liet het systeem veelbelovende resultaten zien en zijn er ook vele mogelijkheden om de bestaande gevoeligheid te verbeteren en zo een directe integratie met het HRS systeem te bereiken.

Een grote stap voorwaarts in HRS zou de mogelijkheid zijn om absolute kwantificering van genomen componenten in een HRS systeem te integreren. Dan zou bijvoorbeeld een kwantitatieve structuur-affiniteitsrelatie (QSAR) gemaakt kunnen worden direct op basis van het mengsel gesynthetiseerd met de beschreven omzettingsmethoden. ICP-MS biedt zich hiervoor aan omdat het heel gevoelig kan zijn en zijn respons vrijwel volledig onafhan-
I. List of publications

Section 2: High-Resolution Screening Platforms


Section 3: Integration of modification methods and HRS workflows for the study of metabolite-like lead libraries


Section 4: Integrated structure elucidation approaches for high-resolution screening


Section 5: High temperature liquid chromatography hyphenated with ESI–MS and ICP–MS detection for the structural characterization and quantification of halogen containing drug metabolites


Other publications, not in the thesis:

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Grade: - Sehr gut (highest grade)
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