Integrated Analytical Strategies for Drug Discovery

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Chapter 7

On-line electrochemistry–bioaffinity screening with parallel HR-LC-MS. 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 p38α kinase inhibitor electrochemical conversion products. This work demonstrates the hyphenation of an electrochemical reaction cell with a continuous-flow bioaffinity assay and parallel LC-HR-MS. Competition of the formed products with a tracer (SKF-86002) that shows fluorescence enhancement in the orthosteric binding site of p38α is the readout for bioaffinity. Parallel HR-MS^n experiments provided information on the identity of binders and non-binders. Finally, the data produced with this on-line system was compared with that of off-line generated electrochemical conversion products. The electrochemical conversion of 1-(6-chloro-5-((2R,5S)-4-(4-fluorobenzyl)-2,5-dimethylpiperazine-1-carbonyl)-3aH-indol-3-yl)-2-morpholinoethane-1,2-dione (DMPIP) resulted in 8 products of which 3 showed bioaffinity in the used continuous-flow bioassay. Electrochemical conversion of BIRB 796 resulted, amongst others, in the formation of the reactive quinoneimine structure and its corresponding hydroquinone. Both products were detected in the 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 the principles and use of on-line electrochemistry-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 be used to eventually 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 is yet available. For many years, we have been developing hyphenated screening assays to obtain chemical and biological information in a combined manner [9].This resulted in several approaches to assess bioaffinity, e.g., on-line receptor binding [10], enzyme activity assessment [11], bacterial growth inhibition [12], as well as several other strategies which allowed us to identify and characterize bioactive compounds [13-15] in (complex) mixtures. These mixtures included natural extracts, crude synthesis products, medicinal chemistry compound libraries, degradation products by light or harsh chemical conditions, and in vitro metabolism studies.

The implementation of a device for electrochemical oxidation in our on-line screening approaches 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, instable and/or reactive products could be analyzed directly after their formation and as such have less change of degradation.

In this paper, we describe the hyphenation of EC with our recently developed liquid chromatography (LC) - on-line p38α mitogen activation protein kinase binding assay with parallel high resolution MS [16]. EC provides relatively clean samples and has shown to facilitate the formation of interesting molecules for drug research [17]. The p38α mitogen activation protein kinase is an prominent example of a drug target kinase [18] and is heavily involved in inflammation processes [19]. The hyphenation of these techniques to develop fully integrated analytical chemical methods can facilitate the hit to lead selection process in drug discovery.

Material 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 of Millipore (Amsterdam, The Netherlands). 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 was obtained from Mallinckrodt Baker (Deventer, the Netherlands). Fused silica tubing (250-µm inner and 375-µm outer diameter) covalently coated with polyethylene glycol (PEG) was obtained from Sigma-Aldrich (Schnelldorf, Germany). Human recombinant p38α, BIRB796, TAK715, 1-(6-chloro-5-((2R,5S)-4-(4-fluorobenzyl)-2,5-dimethylpiperazine-1-carbonyl)-3aH-indol-3-yl)-2-morpholinoethane-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).

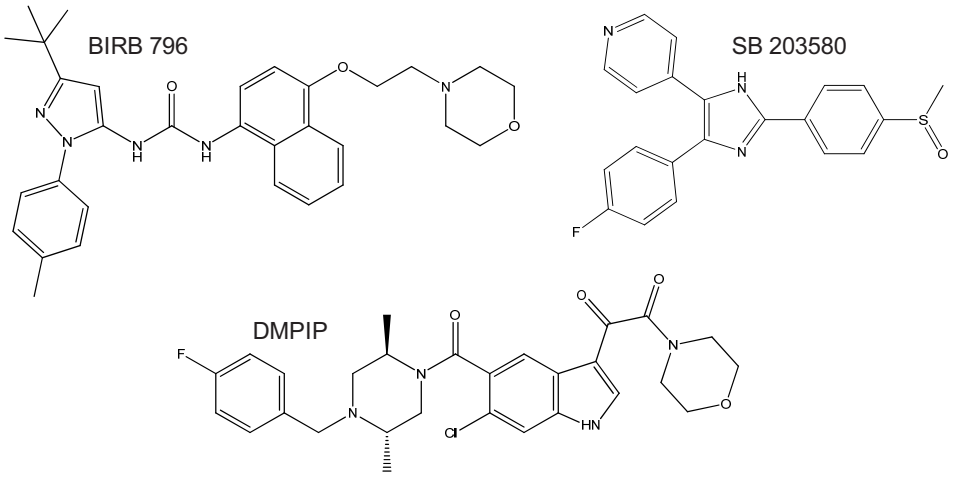

Figure 1: Structures of the kinase inhibitors used for electrochemical conversion experiments.

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.

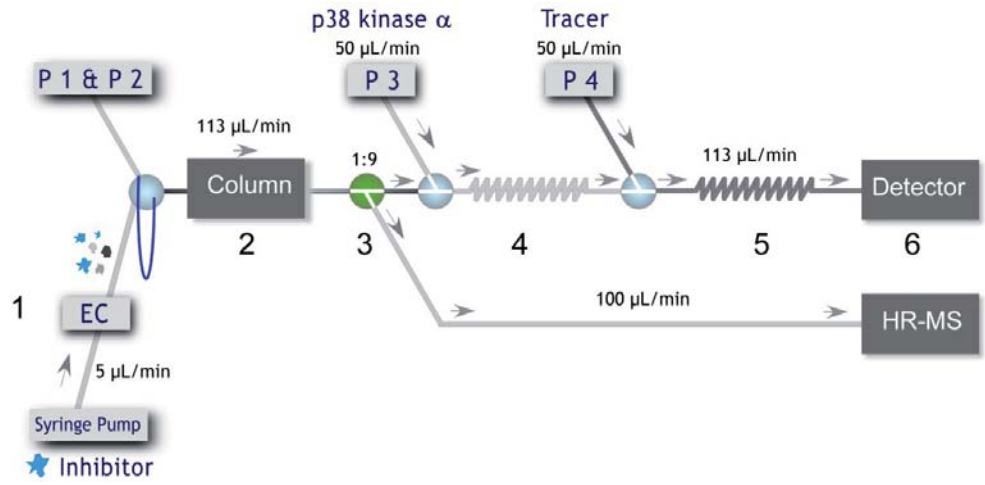

Figure 2: Scheme of the on-line setup (1) online electrochemical conversion of inhibitor (2) Gradient LC separation of products formed (3) split of 1:9 to MS and bioassay (4) reaction coil for enzyme binding (5) addition of tracer molecule (6) detection of enzyme tracer complex by fluorescence and parallel HR-MS^n for structural information of binders.

A Roxy electrochemical reactor 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 µM kinase inhibitor standards, dissolved in 25% ACN and 75% 1 mM aqueous buffer, were infused at a flow rate of 5 µL/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 µL 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 µL PEEK 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, an SIL-10ADvp auto injector, a CTO-10ASvp column oven, a 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 20% B, gradient to 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 µL/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 bioassay 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 6V. 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 µl/min of the LC mobile phase being directed to the bioassay and 100 µl/min to the MS system. The post-column fluorescence enhancement based bioaffinity assay towards the p38 α kinase was identical to the system previously described by us [16]. 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 p38α is the readout for bioaffinity.

Parallel to the p38α kinase 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 with a flow-rate of 1.5 L/min. MS^2 and MS^3 spectra were acquired in data-dependent mode with the following settings: full-spectrum MS with *m/z* 200 – 650, MS^2 with *m/z* 150 – 650 and MS^3 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 was used for structure identification. External calibration of the instrument was based on sodium TFA clusters and allowed a mass accuracy lower than 5 ppm.

Optimization of electrochemical conversion

For flow-through electrochemical cells, important parameters reported are pH, the 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 [5,20]. 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 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 same 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.

Table 1: Optimized electrochemical conversion conditions for DMPIP, BIRB 796 and SB203580.

EC conditions	DMPIP	BIRB 796	SB 203580
pH 3.5	**+ 1.2 V**	**+ 0.8 V**	
pH 5.0			**+ 1.5 V**
pH 10	**- 1.5V** **+ 1.2 V**	**+ 0.8 V**	**+ 1.5 V**

Results and discussion

In this study, an integrated approach is developed for the formation, identification and biological characterization of p38α kinase inhibitor conversion products. This work demonstrates the hyphenation of an electrochemical reaction cell with a continuous-flow bioaffinity assay and parallel LC-HR-MS. As a model system, the recently developed p38α bioaffinity assay based on fluorescence enhancement was used. Parallel HR-MS^n experiments provide information on the identity of binders and non-binders. Finally, the data produced with this on-line system was compared with 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 do not differ in the EC part itself. 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 supporting information 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 can be strongly hampered by ion suppression effects as well as by risk of unwanted in-source fragmentation.

Figure 3 shows an overlay of the bioaffinity traces of on-line electrochemistry-LC-p38α affinity experiments and the relevant extracted ion chromatograms (EIC) from the simultaneous LC-MS experiments for the compound DMPIP ($C_{28}H_{30}ClFN_4O_4$, $[M+H]^+$ 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 four 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 with 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. Please note that, as explained in our previous paper [16], correlating bioaffinity

chromatograms with EIC is more reliably done at the front of the peak rather than at the top.

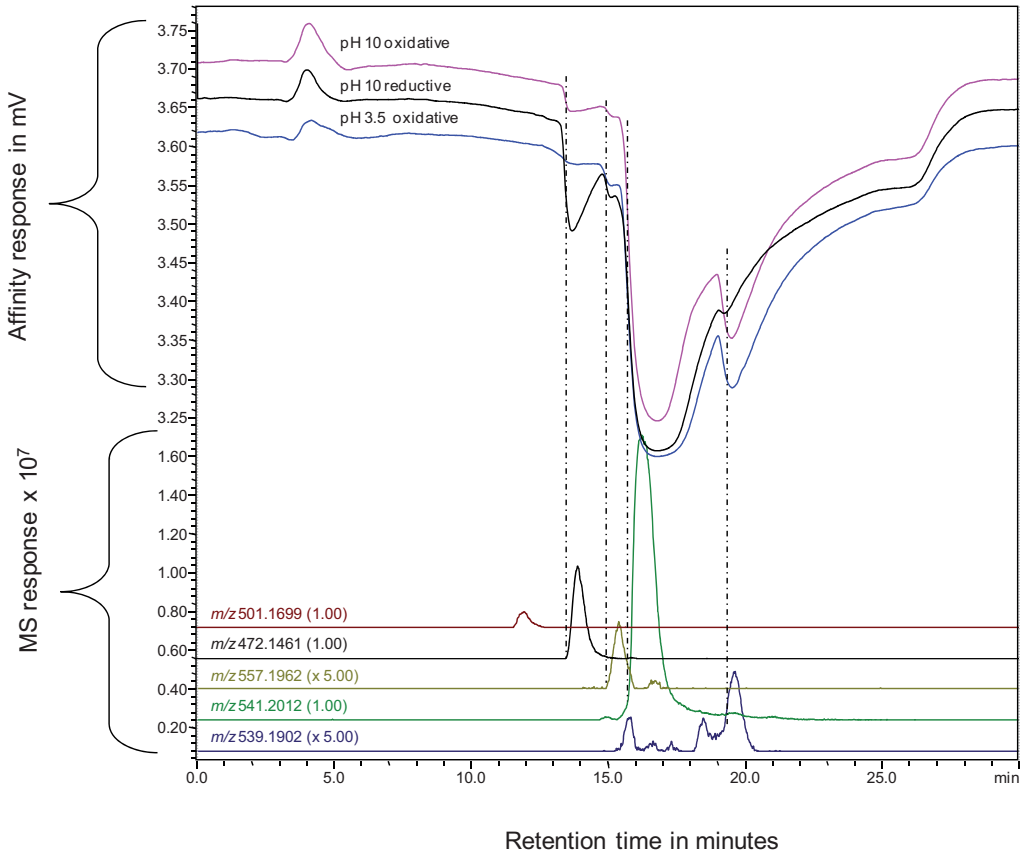

Figure 3: Correlation of bioaffinity profiles (top) and HR-MS traces (bottom) of electrochemical conversion products related to DMPIP.

The major affinity peak is of the unmodified kinase inhibitor, DMPIP. Based on the bioaffinity profile, the product with *m/z* 539.1902 (Δ –2.011 u) showed to be the main bioactive product in oxidative mode. However, in reductive mode this product was less abundant and a product with *m/z* 472.1461 (Δ –69.0551 u) also showed significant binding to the p38α kinase. In both reductive and oxidative mode, a minor affinity peak was observed corresponding to hydroxylated DMPIP, with *m/z* 557.1962 (Δ +15.9950 u).

Identification of the (bioactive) products was done using the HR-MS^n data obtained in parallel with the bioaffinity measurements. Based on the

fragmentation of the unmodified kinase inhibitor, 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 characteristic parts of the molecule involved in the fragmentation reactions.

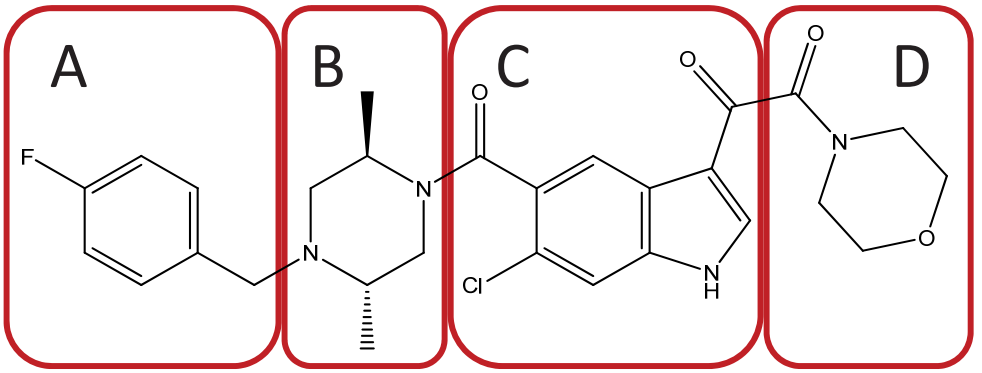

Figure 4: Structure of DMPIP and characteristic parts for MS/MS identification of electrochemical conversion products.

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 results of the identification study.

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 Δ –69.0551 u, consistent with a net loss of C_4H_7N. 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 C_4H_7N. Fragmentation of the $[M+H]^+$ additionally results in de neutral loss of CO_2, leading to an unmodified B+C fragment. The loss of CO_2 indicates the hydrolysis of the amide in part D to a carboxylic acid, which is supported by the MS^3 fragments (data not shown).

Table 2: Identification of electrochemical conversion products related to DMPIP based on high resolution MS^n experiments (for explanation: see text). Binding to p38α is defined as a S/N ratio of > 3 in the bioaffinity assay (see also figure 3).

T_r in min.	$[M+H]^+$	Structure	Molecular formula	MS^n A	B	C	D	other	p38α binder
16.1	541.2012	DMPIP	$C_{28}H_{31}ClFN_4O_4^+$	+B	+A	√	+C		√
9.3	431.1481	- C_7H_6F	$C_{21}H_{25}ClN_4O_4^+$	-	-	√	√		-
11.9	501.1699	- C_3H_4	$C_{25}H_{27}ClFN_4O_4^+$	-	+C -C_3H_4	+D	+C		-
12.8	525.1699	- CH_4	$C_{27}H_{27}ClFN_4O_4^+$	+B - CH_4	+A -CH_4	+D	+C		-
14.0	472.1461	- C_4H_7N	$C_{24}H_{24}ClFN_3O_4^+$	+B	+A	√	-C_4H_7N	-CO_2	√
15.6	557.1962	+ O	$C_{28}H_{31}ClFN_4O_5^+$	+B	+A	√	+O		√
15.6	529.1649	- C_2H_4 + O	$C_{26}H_{27}ClFN_4O_5^+$	√	-C_2H_4 +O	+ D	+C	-CO -H_2O	-
17.5	571.2118	+ CH_2O	$C_{29}H_{33}ClFN_4O_5^+$	-	-	+D	√	-H_2CO	-
19.6	539.1902	- 2H	$C_{28}H_{29}ClFN_4O_4^+$	-	- 2H	+D	+C		√

Other products were identified in a similar way. Oxidation in the D part of DMPIP retained the affinity for the p38α kinase as well as dehydrogenation in the B part, see table 1. Products not showing bioaffinity include methoxylation of part A (*m/z* 571.2110, $C_{29}H_{33}ClFN_4O_5^+$), cleavage of methane from part B (*m/z* 525.1720, $C_{27}H_{27}ClFN_4O_4^+$) and the loss of C_3H_4 from part B (*m/z* 501.1715, $C_{25}H_{27}ClFN_4O_4^+$). The electrochemical induced cleavage of part A resulted in a product with *m/z* 431.1476, $C_{21}H_{24}ClN_4O_4^+$).

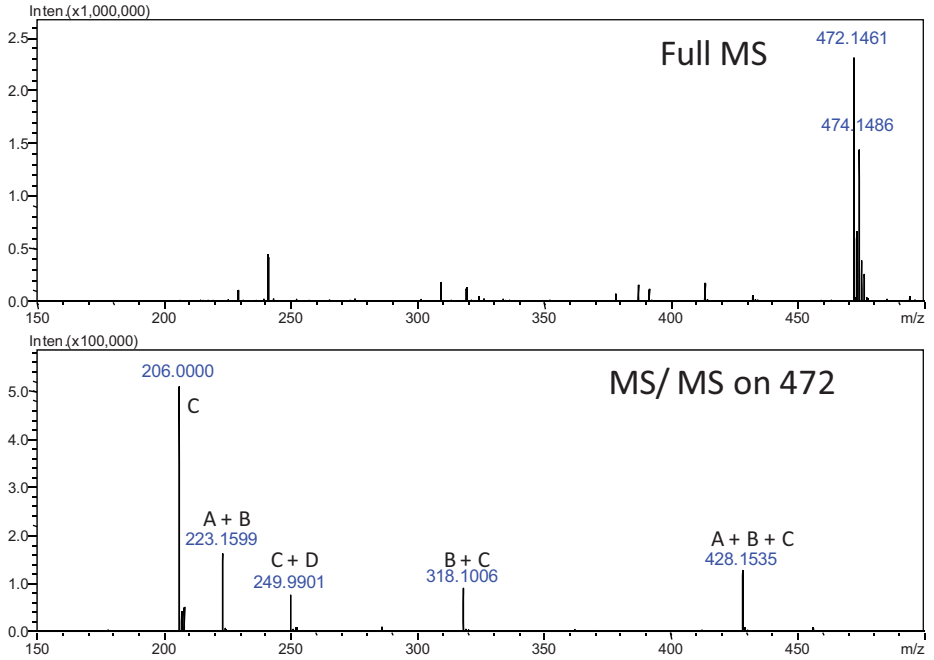

Figure 5: MS and MS/MS spectra of electrochemical conversion product of DMPIP, with m/z 472.1461. Annotations related to the parent structure (figure 4) are included in the spectra.

The bioaffinity assessment of the products based on the BIRB 796 inhibitor ($C_{31}H_{37}N_5O_3$, $[M+H]^+$ with *m/z* 528.2975) showed that next to BIRB 796 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, $C_{25}H_{25}N_4O_2^+$, Δ 115.0990 u) and the hydroquinone (*m/z* 415.2134, $C_{25}H_{27}N_4O_2^+$, Δ 113.0841 u), both being present in all conversion samples.

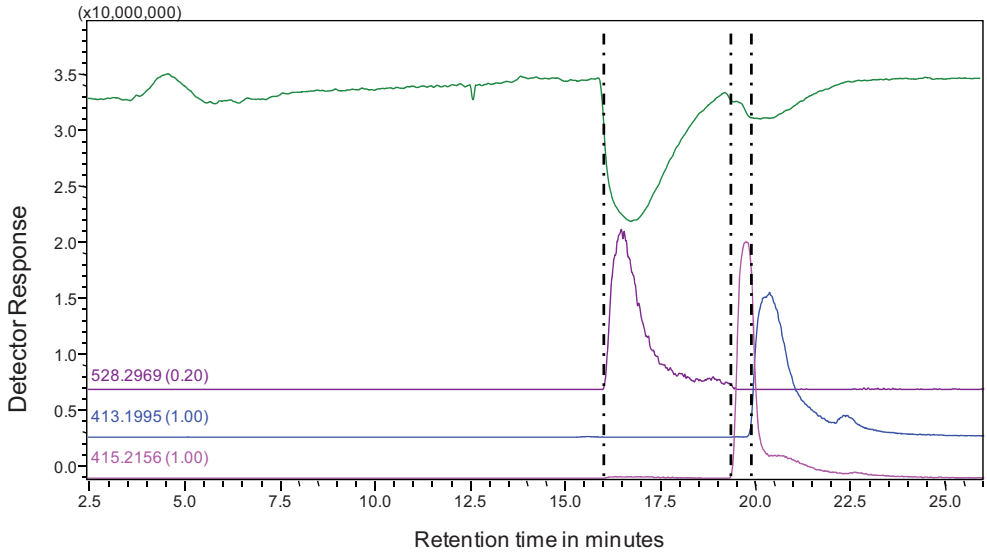

Figure 6: Correlation of bioaffinity profiles (top) and HR-MS traces (bottom) of electrochemical conversion products related to BIRB 796, conversion at pH 3.5 with 0.8 V.

Interestingly, Regan *et al.* described the structure-activity relationships of BIRB 796 and some of its fragments [21]. The hydroquinone was found to bind to the kinase, while the reactive quinoneimine was not addressed at all. 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 BIRB 796 were not bioactive. These included a product with *m/z* 230.1652 corresponding to $C_{14}H_{20}N_3^+$ and a low abundant product with *m/z* 273.1710 corresponding to $C_{15}H_{21}N_4O^+$, co-eluting with the unmodified BIRB 796. Structure proposals can be found in the supporting information figure S1. Oxidation at pH 7.0 and 10.0 resulted in two additional products with *m/z* 386.1863 ($C_{24}H_{24}N_3O_2^+$) and 411.1821 ($C_{25}H_{23}N_4O_2^+$).

For SB203580 and its conversion products, affinity determination was hampered by the auto-fluorescence of SB203580 and its products at the wavelengths used for the detection of the enzyme-tracer complex in the bioassay. Electrochemical conversion of SB203580 ($C_{21}H_{17}FN_3OS^+$, $[M+H]^+$ with *m/z* 378.1071) resulted in the

formation of single (*m/z* 394.1026) and double oxygenated molecules (*m/z* 410.0969). The main product could be identified as the sulphone product based on its fragmentation pattern described previously by Henklova *et al.* [22]. The rather scarce fragmentation of SB203580 did not allow proposing structures of the other products generated by electrochemical conversion.

In order to compare the on-line formation with the conventional off-line generated products, a set of off-line electrochemical conversion experiments were 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. For BIRB 796, differences were observed between off- and on-line conversion products. The peak area of the reactive quinoneimine significantly decreased in the duplicate measurement after 10hrs (see 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 formation, identification and affinity determination strategy 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 quinone imine of the BIRB 796 inhibitor.

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 chamber. 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.

Acknowledgements

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Supporting Information

Figure S1: BIRB 796 related structures.

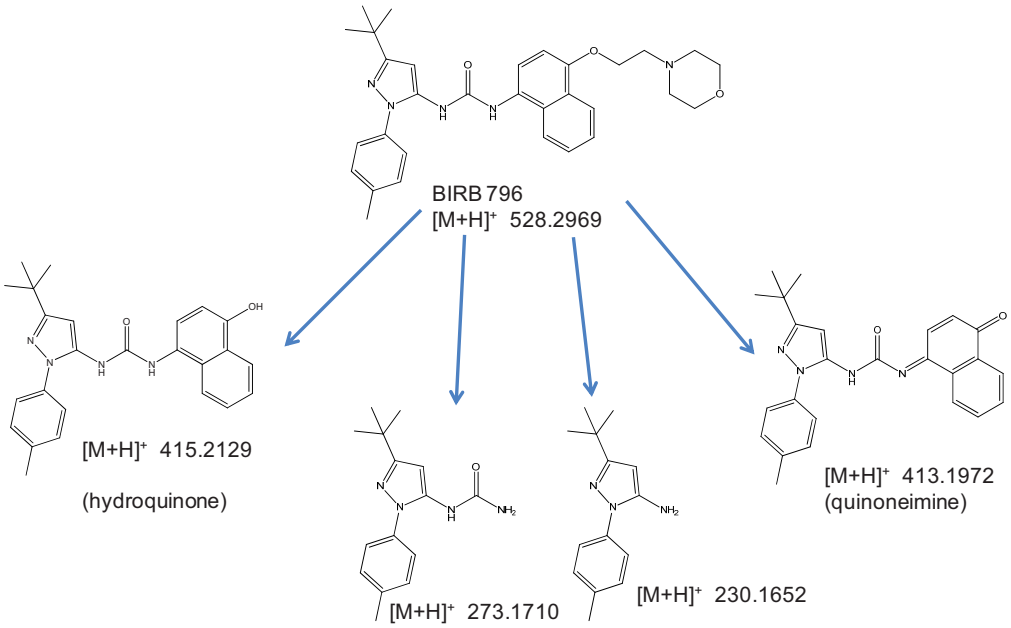

Figure S2: BIRB-796 off-line products, duplicate after 10 hrs