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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 analyzed 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, for example by comparison of obtained IC_{50} values with other formats [5]. It enabled 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 know 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

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the lipophilicity of the inhibitors and may retain 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 (H_2O_2), 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 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 H_2O_2 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 allows us to assess the usefulness of employing 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 an 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 DMPIP (1-{6-chloro-5-[(2R,5S)-4-(4-fluorobenzyl)-2,5-dimethylpiperazine-1-carbonyl]-3aHindol-3-yl}-2-morpholinoethane-1,2-dione), SB203580 (4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine) [23], BIRB796 (N-[3-(*tert*-butyl)-1-(4-methylphenyl)-1H-pyrazol-5-yl]-N′-[4-[2-(4-morpholinyl)ethoxy]-1-naphthalenyl]-urea) [24], and TAK-715 (N-[4-[2-ethyl-4-(3-methylphenyl)-5-thiazolyl]-2-pyridinyl]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^{-1} 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 DMPIP, 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 150 W 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 assess 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 μM p38α inhibitor, 2 mg ml^{-1} 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^{-1} 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