Novel Bioactivation Mechanism of Reactive Metabolite Formation from Phenyl Methyl-Isoxazoles

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ABSTRACT:

Recently, we described a series of phenyl methyl-isoxazole derivatives as novel, potent, and selective inhibitors of the voltage-gated sodium channel type 1.7 (Nav1.7) (Macsari et al., 2011). Some of the most active compounds exhibited IC50 values in the 10 nM range with the lead compound, 2-chloro-6-fluorobenzyl [3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl]-carbamate, showed unprecedented GSH and cysteine reactivity associated with NADPH-dependent metabolism in trapping studies using human liver microsomes. Additional trapping experiments with close analogs and mass spectra and NMR analyses suggested that the conjugates were attached directly to the 5'-methyl on the isoxazole moiety. We propose a mechanism of bioactivation via an initial oxidation of the 5'-methyl generating a stabilized enimine intermediate and a subsequent GSH attack on the 5'-methylene. Efforts to ameliorate reactive metabolite generation were undertaken to minimize the potential risk of toxicity. Formation of reactive metabolites could be significantly reduced or prevented by removing the 5'-methyl, by N-methylation of the carbamate; by replacing the nitrogen with a carbon or removing the nitrogen to obtain a carboxylate; or by inserting an isomeric 5'-methyl isoxazole. The effectiveness of these various chemical modifications in reducing GSH adduct formation is in line with the proposed mechanism. In conclusion, we have identified a novel mechanism of bioactivation of phenyl 5-methylisoxazol-4-yl-aminines. The reactivity was attenuated by several modifications aimed to prevent the emergence of an enimine intermediate. Whether 5'-methyl isoxazoles should be considered a structural alert for potential formation of reactive metabolites is dependent on their context, i.e., 4'-nitrogen.

Introduction

Adverse drug reactions are a serious complication of drug therapy. As a consequence of evidence of toxicity, 13% of approved drugs for the U.S. market between 1975 and 1999 received either a black-box warning or were withdrawn (Park et al., 2011). The formation of reactive metabolites has been implicated in many adverse drug reactions, including organ toxicities, idiosyncratic reactions, and hypersensitivity reactions. Although the latter categories are not necessarily evident in preclinical species and are rare, they can be serious or even fatal in human (Li and Uetrecht, 2010; Thompson et al., 2011).

A common strategy in current drug discovery for trying to avoid these adverse drug reactions is to screen compounds for their propensity to form reactive metabolites and apply chemical modifications that prevent bioactivation. The first step in the risk assessment is done by identification of structural alerts, i.e., risk-filled chemical moieties known to generate reactive metabolites in certain constellations (Stepan et al., 2011). Moreover, high-throughput in vitro assays using hepatic microsomes and, for example, GSH or cysteine (Cys) have been developed to detect stable thioether conjugates that are produced via reactive intermediates (Park et al., 2011; Liao et al., 2012). If the occurring reactivity originates from a part of the molecule that is not part of the pharmacophore, the preferred solution is to replace the perpetrating group and eliminate the liability. If the mechanism of bioactivation involves part molecule that is essential for activity, it can be more complicated. In these instances, efforts may be directed to balance between high efficacy (resulting in a low human dose) and reduced generation of reactive metabolites. The latter may be achieved by improving the overall metabolic stability of the drug candidates or by redirecting metabolism to less harmful locations in the molecule by blocking specific identified metabolic paths or introducing a metabolic soft spot. It has been suggested that idiosyncratic reactions are dose dependent and do not typically occur when the dose is as low as 10 mg per day, and therefore optimizing efficacy could potentially mitigate risks with reactive bioactivation (Uetrecht, 1999; Obach et al., 2008).

Recently, we described the identification of a novel phenyl methyl-isoxazole series as potent inhibitors of the voltage-gated sodium channel type 1.7 (Na+, 1.7) (Macsari et al., 2011). Some of the most active compounds exhibited IC50 values in the 10 nM range with
promising selectivity toward Na, 1.5. Selectivity against Na, 1.5 is a prerequisite, because this sodium channel fulfills an essential role in heart physiology and cannot be tampered with (Rook et al., 2012). However, the phenyl isoxazole series suffered from poor metabolic stability and solubility. The lead compound 1, 2-chloro-6-fluorobenzyl [3-(2,6-dichlorophenyl)-5-methylisoxazol-4-y]carbamate (Fig. 1; Table 1), showed unprecedented GSH reactivity in initial GSH trapping experiments.

Several drugs, including valdecoxib (Fig. 1), parecoxib, oxacillin (Fig. 1), dicloxacillin, cloxacillin, and fluoroxacillin, also contain this phenyl methyl-isoxazole fragment. Serious skin reactions, including erythema multiforme, Stevens-Johnson syndrome, and toxic epidermal necrolysis, have been reported (Olsson et al., 1992; Andrews and Daly, 2008). Liver injuries have been observed, as well as possible immunohallergic idiosyncratic reactions. Again, the underlying mechanism of toxicity remains to be clarified.

The aim of the present studies was to elucidate the underlying mechanism of reactive metabolite formation of the phenyl methyl-isoxazole series. Furthermore, we investigated synthetic modifications of the lead compounds to avoid generation of reactive metabolites to continue developing this series as potent and selective Na, 1.7 channel blockers, but minimize the potential risk of toxicity.

Materials and Methods

Chemicals and Reagents. Test compounds originated from the compound collection of AstraZeneca R&D, and the synthesis of such derivatives has been described by Macsari et al. (2011), with the exception of the isopropyl phenyl 5’-methylisoxazole analogs (compounds 18 and 19) that are described in the supplemental data (Supplemental Figs. 1 and 2). GSH, Cys, and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). Samples of human liver (HL53, HL55, HL59, HL60, HL71, HL73, and HL75) were obtained from the liver bank of Huddinge Hospital. Liver microsomes were isolated from these seven different individuals as described previously (Löfger et al., 2004). All standard chemicals used for in vitro studies were purchased from reputable companies and were of standard purity. Solvents used for liquid chromatography were of gradient grade purity and bought from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q water purification system (Millipore Corporation, Billerica, MA). LC-MS analysis was carried out in 12 independent 2-ml incubations, which each contained 100 μM substrate and 1 mg/ml HLM. The incubations were placed at 37°C under gentle shaking and terminated after 45 min by the addition of 2 volumes of acetonitrile. The incubations were pooled and centrifuged (20,000g for 5 min), and the supernatant was evaporated. The residue was reconstituted with 10% acetonitrile in water, and the Cys adduct-containing solution was loaded on a SPE column (OASIS HLM 20cc; Waters), eluted with acetonitrile in water and evaporated by lyophilization. The purified metabolite was dissolved in 100 μl of DMSO-d6 and transferred to a 2.5-mm NMR tube.

NMR spectra of compound 6 (2 mg in 100 μl of methanol-d4 in a 2.5-mm NMR tube) and the Cys adduct of compound 6 were recorded on a DRX600 MHz spectrometer (Bruker, Newark, DE) equipped with a 2.5-mm TXI probe (Bruker) with 2 gradients. The residual proton solvent signal was used as a proton reference and set to 3.31 or 2.51 ppm for methanol-d4 (solvent A) and 0.1% formic acid in water/acetonitrile (98:2), v/v (solvent A) and 0.1% formic acid in water/acetonitrile (20:80), v/v (solvent B). Mass spectrometry was performed on a LTQ linear ion trap (Thermo Fisher Scientific, Waltham, MA). An electrospray interface in either the positive or the negative ion mode was used in all experiments. The following scan events were used: event 1, survey scan (120–900 atomic mass units); event 2, data-dependent scan, product ion scans of the most intense ion from scan event 1; event 3, product ion scan of the most intense product ion from scan event 2; event 4, product ion scan of the second-most intense product ion from scan event 2. The software Xcalibur 2.0 (Thermo Fisher Scientific) was used for data acquisition, processing, and control of the mass spectrometer.

Results

In Vitro Metabolism of Carbamate and Urea Leads. The phenyl methyl-isoxazole compounds exhibited low metabolic stability in HLMs with C10 values generally exceeding 100 μM · min⁻¹ · mg protein⁻¹ (Macsari et al., 2011). Compound 6 was shown as a typical

![Fig. 1. Structural formula of compounds discussed in the text.](image-url)
In vitro trapping of initial phenyl methyl-isoxazole carbamate and urea leads

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Example from all first-wave analogs of both the carbamate (compounds 1, 4, 5, 8, and 9) and the urea (compounds 2, 3, 6, and 7) subseries (Table 1). The $C_{\text{int}}$ value for compound 6 was $>500 \mu l \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and the metabolism occurred in a NADPH-dependent manner (Fig. 2, A and B). Trace amounts of detectable oxidative metabolites, which did not quantitatively account for the disappearance of the parent compound, were detected using liquid chromatography tandem mass spectrometry analysis with either positive (Fig. 2B) or negative electrospray ionization. However, a major metabolite with an increased $m/z$ value of 305 was identified using GSH as a trapping reagent (Fig. 2C). Interpretation of product ions mass spectra of the parent compound and the major metabolite formed in GSH trapping experiments suggested that the GSH adduct was associated with the phenyl methyl-isoxazole part of the molecules (Fig. 3). The interpretation of the proposed fragmentation as depicted in Fig. 3 was further supported by the chloride isotope pattern (data not shown). Compound 10 formed a GSH adduct with the same increase in $m/z$ value and strengthened the mass spectrum interpretation. Trapping experiments using Cys yielded a similar metabolic profile represented by a major adduct of an increased $m/z$ value of 119, which is in agreement with the insertion of Cys. Tandem MS analysis suggested that the localization of the Cys adduct also was associated with the phenyl methyl-isoxazole part of the molecules (data not shown).

Identification of Adduct Localization. However, the exact position of the GSH and Cys conjugates could not be ascertained by mass spectrometry alone. This information was of interest to guide the synthesis of new, potentially more stable, and less reactive compounds. It has been suggested that phenyl moiety epoxidation for the structural analog valdecoxib caused GSH adduct formation in the mouse (Zhang et al., 2003). However, analysis of trapping experiments for some analogs e.g., compound 8, made that assumption questionable for the phenyl methylisoxazoles. An aromatic epoxidation of a fluoride-substituent phenyl molecule often generates, at least partially, a GSH adduct with an increased $m/z$ value of 303 due to loss of the fluoride atom (Samuel et al., 2003). No such GSH adduct could be detected in HLM trapping experiments aside from the GSH adduct with an increased $m/z$ value of 305. Therefore, the Cys adduct of compound 6 was produced, isolated, and subjected to NMR analysis (Fig. 4).

The proton spectrum revealed that the CH$_3$ was replaced by a CH$_2$ in the Cys adduct and showed nonequivalent protons and no neighboring (J-coupled) protons. This implies that Cys is directly attached to the newly formed CH$_2$ group. The predicted and observed proton chemical shifts were 3.89 and 3.86 ppm, respectively. The location of the Cys conjugate was further verified using correlations in the nuclear Overhauser effect spectroscopy and total correlation spectroscopy spectra and comparisons with the proton spectrum of the parent compound (Supplemental Figs. 3–10). In summary, the NMR analysis concluded that the Cys adduct was directly attached to the 5'-methyl on the isoxazole moiety.

Resolving Reactive Metabolite Formation. The localization of the Cys adduct directly to the methyl group of the isoxazole indicated formation of a reactive metabolite with the 5'-methyl group as a reactive center. We propose a mechanism of bioactivation initiated by cytochrome P450-mediated 5'-methyl oxidation yielding a stabilized enamine intermediate either directly or via a hydroxylated intermediate. The enamine is then proposed to react with GSH at the methylene, leading to the final detected metabolite (Fig. 5).

Because the isoxazole moiety was part of the pharmacophore, efforts to ameliorate reactive metabolite generation without affecting potency or selectivity were undertaken to minimize the potential risk of toxicity. Three approaches to reduce or eliminate the reactive metabolite formation were undertaken: 1) removal or modification of the 5’-methyl group; 2) removal or changing the 4’-nitrogen function-
ality; and 3) insertion of isomeric methyl isoxazoles to eliminate formation of a potential reactive enamine intermediate (Table 2).

Trapping experiments showed that no GSH adducts were detected for compounds 11 and 12 that lacked the 5'-methyl group. However, Na<sub>v</sub> 1.7 potency was not ideal for these compounds. Attempts to replace the 5'-methyl by trifluoromethyl, cyano, or chloro failed due to synthetic difficulties. Compounds 13 and 14 demonstrated that GSH conjugate formation was abolished when the 4-nitrogen was exchanged with a carboxylate. Also N-substitution of the 4'-nitrogen with a methyl group (15), blocked the GSH adduct formation. However, these structural modifications also led to less potent compounds (Macsari et al., 2011). Therefore, we turned our attention to carboxamides, derivatives where the nitrogen is replaced by a carbon (compounds 16 and 17). This modification would also decrease the likelihood of reactive metabolite formation, because a putative cationic intermediate formed by metabolic activation would be less stabilized.

Indeed, only relatively small amounts of GSH adducts were detected in trapping experiments with compounds 16 and 17. Both compounds formed low, but detectable, amounts of a GSH adduct with the increased m/z value of 305. In addition, compound 16 formed a GSH adduct with an increased m/z value of 321, which likely represents GSH conjugation in combination with hydroxylation. However, the trapping data strongly suggested that the subseries had been improved with respect to formation of reactive metabolites. Figure 6 shows metabolic profiling in HLM incubates supplemented with NADPH and GSH of a representative compound of the urea subseries (compound 7) and the carboxamide analogs 16 and 17.

All three compounds were found to be metabolically unstable with Cl<sub>int</sub> values of >100 µl·min<sup>-1</sup>·mg protein<sup>-1</sup> in HLMs. The metabolic patterns differed dramatically with respect to the detection of oxidized products. Whereas the urea analog almost exclusively was converted into a single GSH adduct (Fig. 6A), several major oxidation products were detected for compounds 16A, 17 (Fig. 6B and C).

The third approach of addressing reactive metabolite formation was to synthesize isomeric methyl isoxazoles. This new structural class of

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**Fig. 2.** LC-MS analysis of formed metabolites in HLM incubates of compound 6. A, control incubation without NADPH supplementation. B, incubation with NADPH. C, trapping incubation with NADPH and GSH supplementation. Chromatograms are showing the combined base peak intensities of m/z 376 (parent); m/z 374, 390, 392, 406, 408, and 410 (oxidized metabolites); and m/z 681 (GSH adduct). P, parent molecule; Ox, oxidized metabolite. Relative abundance values are presented for each individual subfigure.

**Fig. 3.** MS/MS interpretation. A, MS/MS spectra of m/z 376 representing the molecular ion (M+H) of the parent molecule (compound 6). B, MS/MS spectra of m/z 681 representing the molecular ion (M+H) of the GSH adduct formed in HLM supplemented with GSH and NADPH. The m/z 552 and m/z 577 signals likely represent the characteristic loss of glutamine (~129 Da) from the GSH substituent itself. Proposed ion fragmentation pattern is shown in insets of the molecular structures.
Nav1.7 antagonists exhibited potencies and selectivity toward Nav1.5, which were slightly worse than the urea and carboxamide subseries (P. E. Lund, E. Venyike, and A. B. Eriksson, unpublished data). No GSH adducts were detected in trapping experiments with compounds 18 and 19.

Discussion

Trapping experiments in HLMs showed that both the 5'-methyl and the 4'-nitrogen were essential for extensive GSH adduct formation and supported the proposed mechanism for bioactivation (Fig. 5). To our knowledge, such a bioactivation of 4'-nitrogen-5-methyl isoxa-
A metabolic pathway for the generation of reactive metabolites was recently described for 3,4-unsubstituted isoxazoles after an enzyme-catalyzed cleavage of the ring to form an α-cyanoenol followed by a condensation with formaldehyde to yield a reactive cyanoacrolein derivative (Yu et al., 2011). However, Kalkutgar et al. (2003) demonstrated using leflunomide and 3-methylleflunomide that the unsubstituted 3'-position was important for this ring opening to happen. Thus, the mechanism of reactive metabolite formation described here is distinct from the one presented here for the phenyl 5'-methyl isoxazoles (Kalgutkar et al., 2003). For somewhat related 3'-methyl-isothiazoles, reactive metabolites have been reported that were not associated with ring scission (Teffera et al., 2010). The methyl was oxidized in a similar manner to the phenyl 5'-methyl isoxazoles described here. However, this metabolite seemed not to be involved in the mechanism leading to GSH adduct formation. Instead, the formation of GSH conjugates is thought to be mediated via oxidation of the sulfur that leads to the alleged reactive methylisothiazole sulfoxide.

Several drugs, including valdecoxib, parecoxib, oxacillin, dicloxacillin, cloxacillin, and flucloxacillin, contain this phenyl methyl-isoxazole fragment. Idiosyncratic and drug-induced liver injury have been reported for these classes of compounds through postmarketing surveillance in patients (Olsson et al., 1992; Ziemer et al., 2007; Andrews and Daly, 2008). The underlying reason for these adverse reactions is at present unknown, but it has been speculated that reactive metabolite species are involved (Park et al., 2011). Whether this is the case or not, these reactive species cannot be generated via the novel mechanism presented here, because these drugs lack the essential 4'-nitrogen.

Sulfamethoxazole (Fig. 1), a sulfonamide containing a 5-methylisoxazol-3-yl moiety, has been associated with many hypersensitivity reactions in several patient populations. It has been postulated that bioactivation of the parent drug to a chemically reactive intermediate is an important step in the development of toxicity (Carr et al., 1993). Several studies have demonstrated metabolism-dependent activation of sulfamethoxazole to reactive metabolites (Rieder et al., 1988; Riley et al., 1991; Carr et al., 1993). In these studies, hydroxyl and nitroso species were identified as the reactive and potentially toxic species, rather than the isoxazole moiety.

In conclusion, we have identified a novel mechanism of bioactivation of phenyl 5-methyl-isoxazol-4-yl-amines. The reactivity was attenuated by several modifications aimed to prevent the emergence

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**TABLE 2**

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*Trace amounts of a GSH adduct with an increased m/z value of 303 were detected. This adduct is likely formed via aromatic epoxidation and subsequent loss of fluoride occurring on the molecule’s right side.
of an enamine intermediate. Whether 5'-methyl isoxazoles should be considered a structural alert for potential formation of reactive metabolites is dependent on their context, i.e., 4'-nitrogen.

Authorship Contributions

Participated in research design: Bylund, Petersson, and Bueters.

Conducted experiments: Bylund, Petersson, Olofsson, and Arvidsson.

Contributed new reagents or analytic tools: Macsari and Besidski.

Prepared data analysis: Bylund, Olofsson, and Bueters.

Wrote or contributed to the writing of the manuscript: Bylund, Macsari, Besidski, Olofsson, Petersson, Arvidsson, and Bueters.

References


