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

Johan Bylund, Istvan Macsari, Yevgeni Besidski, Susanne Olofsson, Carl Petersson, Per I. Arvidsson and Tjerk Bueters

Uppsala University, Department of Biosciences, Uppsala Biomedical Centre, Uppsala University, Box 591, SE-751 24 Uppsala, Sweden. (JB)
Organic Pharmaceutical Chemistry, Department of Medicinal Chemistry, Uppsala Biomedical Centre, Uppsala University, Box 574, SE-751 23 Uppsala, Sweden. (PIA)
School of Pharmacy and Pharmacology, University of KwaZulu-Natal, Durban 4001, South Africa. (PIA)
Running title:
Reactive metabolism of phenyl methyl-isoaxazoles

Corresponding author:
Johan Bylund. Phone: +46-8-55327976. Fax: +46-8-55321560.
E-mail: Johan.Bylund@astrazeneca.com.

# of pages: 26
# of tables: 2
# of figures: 6
# of references: 28
# of words in abstract: 233
# of words in introduction: 611
# of words in discussions: 472

Abbreviations:

Cl_{int}: intrinsic clearance; COSY: Correlation spectroscopy; CYP: Cytochrome P450; DMSO: Dimethyl sulfoxide; ESI: Electrospray ionization; HLM: Human liver microsomes; HMBC: Heteronuclear multiple bond correlation; HSQC: Heteronuclear single quantum coherence; LC-MS: Liquid chromatography mass spectrometry; LC-MS/MS: Liquid chromatography tandem mass spectrometry; N_{av}: voltage-gated sodium channel; NOE: Nuclear Overhauser Effect; NOESY: Nuclear Overhauser effect spectroscopy; TOCSY: Total correlation spectroscopy
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 [Macsari et al. (2011) Bioorg Med Chem Lett 21:3871-76]. The lead compound, 2-chloro-6-fluorobenzyl [3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl]carbamate, showed unprecedented glutathione (GSH) and cysteine (Cys) 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 enamine intermediate and a subsequent GSH attack on the 5’-methylene. Efforts to ameliorate reactive metabolite generation were undertaken in order to minimize the potential risk of toxicity. Formation of reactive metabolites could be significantly reduced or prevented by removing the 5’-methyl, N-methylation of the carbamate, 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-methyl-isoxazol-4-yl-amines. The reactivity was attenuated by several modifications aimed to prevent the emergence 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.
Introduction

Adverse drug reactions are a serious complication of drug therapy. As a consequence of evidence of toxicity, 13% of approved drugs for the US 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 Cys have been developed to detect stable thioether conjugates that are produced via reactive intermediates (Liao et al., 2012, Park et al. 2011). 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 are 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 (Obach et al. 2008; Uetrecht 1999).

Recently, we described the identification of a novel phenyl methyl-isoxazole series as potent inhibitors of the voltage-gated sodium channel type 1.7 (Nav 1.7) (Macsari et al., 2011). Some of the most active compounds exhibited IC_{50} values in the 10 nM range with promising selectivity towards Nav 1.5. Selectivity against Nav 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). The phenyl isoxazole series suffered, however, from poor metabolic stability and solubility. The lead compound 1, 2-chloro-6-fluorobenzyl [3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl]carbamate (Figure 1 and Table 1), showed unprecedented GSH reactivity in initial GSH trapping experiments.

Several drugs including valdecoxib (Figure 1), parecoxib, oxacillin (Figure 1), dicloxacillin, cloxacillin and flucloxacillin also contain this phenyl methyl-isoxazole fragment. Serious skin reactions, including erythema multiforme, Stevens-Johnson syndrome, and toxic epidermal necrolysis, have been reported through postmarketing surveillance in patients receiving valdecoxib, although it is at present unknown what the root cause to these adverse drug reactions is (Ziemer et al., 2007). Even for the oxacillin derivatives adverse drug reactions have been reported (Andrews and Daly, 2008; Olsson et al., 1992). Liver injuries have been observed, as well as possible immunoallergic 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 in order 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 AstraZeneca’s compound collection and the synthesis of such derivatives have been described in Macsari et al., 2011, with the exception of the isomeric phenyl 5’-methylisoxazole analogues (compound 18 and 19) that are described in the supplemental data section (Supplemental Figures 1 and 2). GSH, Cys, NADPH were obtained from SigmaAldrich (St. Louis, Mo). Samples of human liver, HL53, 55, 59, 60, 71, 73 and 75, were obtained from the liverbank of Huddinge Hospital. Liver microsomes were isolated from these seven different individuals as described (Löfgren 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, Bedford, MA). NMR solvents used were 99.8% ampoule DMSO-d6 or Methanol-d4 (Cambridge Isotope Laboratories Inc.).

In vitro metabolic experiments

Microsome incubations for Clint determinations and metabolic profiling experiments were conducted as previously described (Sohlenius-Sternbeck et al., 2010). Briefly, microsomes at concentrations 0.5 mg protein/ml supplemented with 1 mM NADPH were incubated at 37°C with gentle shaking in a 5% CO2 atmosphere. Trapping experiments were conducted in the same way with 10 µM substrate concentrations and final concentrations of 5 mM GSH or 5 mM Cys. All incubations were stopped by the addition of two volumes of ice-cold acetonitrile and were then centrifuged prior to LC-MS/MS analysis. The metabolic capacity of the microsomes was checked in-house using a set of probe substrates. Clint values were obtained from disappearance curves of
the parent molecule (Floby at el., 2009). Compound 6 was included in all subsequent trapping studies as a positive control and reference.

**LC-MS analysis**

LC-MS analysis for Cl\textsubscript{int} value estimations was performed with a Micromass Quattro Micro triple quandropole as previously described (Floby at el., 2009), whereas LC-MS analysis for trapping experiments were carried out as described below. Liquid chromatography was performed with Schmidazu LC-10AD VP pumps (Schimadzu Deutschland, Duisburg, Germany) and a CTC HTC PAL. The separations were performed on a Waters atlantis T3 column (100 x 2.1 mm ID, 3 µm particle size) at a flow rate of 0.25 mL/min. The mobile phase was a binary mixture of 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 Electron Corporation, San Jose, CA, USA). 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 amu. *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 Electron Corporation) was used for data acquisition, processing and control of the mass spectrometer.

**NMR analysis**

Generation of the Cys adduct of compound 6 for NMR analysis was carried out in 12 independent 2 ml incubations which each contained 100 µM substrate and 1mg/ml HLM. The incubations were placed at 37°C under gentle shaking and terminated after 45 minutes by the
addition of two volumes of acetonitrile. The incubations were pooled and centrifuged (20,000 x g for 5 minutes) 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 (Waters OASIS HLM 20cc), eluted with acetonitrile in water and evaporated by lyophilization. The purified metabolite was dissolved in 100 µl DMSO-d6 and transferred to a 2.5 mm NMR tube.

NMR spectra of compound 6 (2 mg in 100 µl Methanol-d4 in a 2.5 mm NMR tube) and the Cys adduct of compound 6 were recorded on a Bruker DRX600 MHz spectrometer equipped with a Bruker 2.5 mm TXI probe with Z gradients. The residual proton solvent signal was used as proton reference and set to 3.31 or 2.51 ppm for Methanol-d3 or DMSO-d5 respectively. The carbon signal from Methanol-d4 was used as carbon reference and set to 49.15 ppm. Spectra were recorded at temperatures between 295 and 300 K. The structure of 6 was verified and resonances assigned using 1D proton, selective 1D NOE, COSY, HSQC and HMBC experiments. The structure of Cys substituted 6 was investigated using 1D proton, NOESY and TOCSY experiments, chemical shift predictions (ACDlabs v9.06) and comparison with the parent compound 6. Additional methodological descriptions are available in the supplemental section.
Results

In vitro metabolism of carbamate and urea leads

The phenyl methyl-isoxazole compounds exhibited low metabolic stability in HLM with Cl_int values generally exceeding 100 µl/min/mg protein (Macsari et al., 2011). Compound 6 is shown as a typical example from all first wave analogs of both the carbamate (compounds 1, 4, 5, 8 and 9) and the urea subseries (compounds 2, 3, 6 and 7) (Table 1). The Cl_int value for compound 6 was >500 µl/min/mg protein and the metabolism occurred in a NADPH-dependent manner (Figure 2A and B). Trace amounts of detectable oxidative metabolites which did not quantitatively account for the disappearance of the parent compound were detected using LC-MS/MS analysis with either positive (Figure 2B) or negative electrospray ionization (ESI). However, a major metabolite with an increased m/z value of 305 was identified using GSH as a trapping reagent (Figure 2C). Interpretation of product ion 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 (Figure 3). The interpretation of the proposed fragmentation as depicted in Figure 3 was further supported by chloride isotope pattern (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 (not shown).

Identification of adduct localization

The exact position of the GSH and Cys conjugates, however, could not be ascertained by mass spectrometry alone. This information was of interest in order 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 rat (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 increase \( m/z \) value of 303 due to loss of the fluoride atom (Samuel at 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 (Figure 4).

The proton spectrum revealed that the \( \text{CH}_3 \) was replaced by a \( \text{CH}_2 \) in the Cys adduct, and showed non-equivalent protons and no neighboring (J-coupled) protons. This implies that Cys is directly attached to the newly formed \( \text{CH}_2 \) 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 NOESY and TOCSY spectra, and comparisons with the proton spectrum of the parent compound (Supplemental Figures 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 5’-methyl group as a reactive center. We propose a mechanism of bioactivation initiated by cytochrome P450 (CYP) 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 (Figure 5).
Because the isoxazole moiety was part of the pharmacophore, efforts to ameliorate reactive metabolite generation without affecting potency or selectivity were undertaken in order 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 functionality and 3) insertion of isomeric methyl isoxazoles in order to eliminate formation of a potential reactive enimine intermediate (Table 2).

Trapping experiments showed that no GSH adducts were detected for compounds 11 and 12 that lacked the 5’-methyl group. Na, 1.7 potency, however, 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. These structural modifications, however, 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, as 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 compound 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 sub-series had been improved in respect to formation of reactive metabolites. Figure 6 shows metabolic profiling in
HLM incubates supplemented with NADPH and GSH of a representative of the urea subseries (compound 7) and the carboxamide analogs 16 and 17. All three compounds were found to be metabolically unstable with Clo values of >100 µl/ml/mg protein in HLM. The metabolic patterns differed dramatically in respect to the detection of oxidized products. Whereas the urea analog almost exclusively was converted into a single GSH adduct (Figure 6A), several major oxidation products were detected for compound 16 and 17 (Figure 6B and C).

The third approach of addressing reactive metabolite formation was to synthesize isomeric methyl isoxazoles. This new structural class of Nav1.7 antagonists exhibited potencies and selectivity toward Na,1.5, which were slightly worse than the urea and carboxamide subseries (unpublished data). No GSH adducts were detected in trapping experiments with compounds 18 and 19.

Discussion

Trapping experiments in HLM showed that both the 5'-methyl and the 4'-nitrogen were essential for extensive GSH adduct formation and supported the proposed mechanism for bioactivation (Figure 5). To our current knowledge, such a bioactivation of 4'-nitrogen-5-methyl isoxazoles have not been reported previously. However, certain drugs such as trimetoprim and dasatinib have been proposed to generate GSH-adducts through enimine intermediates (Lai et al., 1999; Li et al., 2009).

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
However, Kalkutgar et al. 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 (Tefera et al., 2010). The methyl was oxidized in a similar manner to the phenyl 5’-methyl isoxazoles described here. This metabolite appeared, however, not to be involved in the mechanism leading to GSH adducts 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 (Andrews and Daly 2008; Ziemer et al., 2007; Olsson et al., 1992). 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, since these drugs lack the essential 4’-nitrogen.

Sulfamethoxazole (Figure 1), a sulfonamide containing a 5-methyl-isoxazol-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-arnines. 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.

**Authorships contribution**

*Participated in research design:* Johan Bylund, Carl Petersson and Tjerk Bueters

*Conducted experiments:* Johan Bylund, Carl Petersson, Susanne Olofsson and Per Arvidsson

*Contributed new reagents or analytical tools:* Istvan Macsari and Yevgeni Besidski

*Performed data analysis:* Johan Bylund, Susanne Olofsson and Tjerk Bueters

*Wrote or contributed to the writing of the manuscript:* Johan Bylund, Istvan Macsari, Yevgeni Besidski, Carl Petersson, Susanne Olofsson, Per Arvidsson and Tjerk Bueters
References


prediction of clearance from hepatocyte and microsome intrinsic clearance for 52 drug compounds. *Xenobiotica* 40: 637-649.


Figure Legends

Figure 1. Structural formula of compounds discussed in the text.

Figure 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, 410 (oxidized metabolites) and \( m/z \) 681 (GSH adduct). P=Parent molecule and Ox=oxidized metabolite. Relative abundance values are presented for each individual sub-figure.

Figure 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 \( m/z \) 377 signals likely represent the characteristic loss of Glutamine (-129 Da) from the GSH substituent itself. Proposed ion fragmentation patter is shown in inserts of the molecular structures.

Figure 4. Localization of adduct formation. A) LC-MS chromatogram of a large scale Cys trapping incubation with compound 6. The chromatogram is showing the combined base peak intensities of \( m/z \) 166-600. Peak representing the parent molecule is marked P and the Cys adduct is marked Cys. B) Structure and numbering of Cys adduct of compound 6. C) Proton NMR spectrum with assignments of the Cys adduct of compound 6. Spectra were recorded in DMSO-d6 at 318 K. Detailed NMR data is available in the supplemental data section.
Figure 5. Proposed mechanism of GSH adduct formation

Figure 6. GSH trapping incubations in HLM of analogs representing the urea and the carboxamide subseries. A) LC-MS chromatogram of compound 7, a representative urea subseries compound. B) LC-MS chromatogram of compound 16. C) LC-MS chromatogram of compound 17. Black trace representing incubates supplemented with NADPH and red traces representing control experiments without NADPH supplementation. The chromatograms showing the combined base peak intensities of the molecular ion (M+H) of the parent molecules m/z 388 (7), m/z 434 (16), and m/z 474 (17) plus the addition of -2, 14, 16, 30, 32 and 34 Da (representing oxidized metabolites), m/z 305 Da (representing GSH-conjugation) and m/z 321, 323, and 337 Da (representing the combination of GSH-conjugation and oxidation).
### Table 1. *In vitro* trapping of initial phenyl methyl-isoxazole carbamate and urea leads

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>GSH adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>YES</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>YES</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>YES</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>YES</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>YES</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>YES</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>YES</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8.png" alt="Structure 8" /></td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>YES</td>
</tr>
<tr>
<td>10</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>YES</td>
</tr>
</tbody>
</table>
Table 2. *In vitro* trapping experiments of modified Na,1.7 antagonist of the phenyl methyl-isoazole series

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>GSH adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td><img src="image1" alt="Structure 11" /></td>
<td>NO</td>
</tr>
<tr>
<td>12</td>
<td><img src="image2" alt="Structure 12" /></td>
<td>NO</td>
</tr>
<tr>
<td>13</td>
<td><img src="image3" alt="Structure 13" /></td>
<td>NO</td>
</tr>
<tr>
<td>14</td>
<td><img src="image4" alt="Structure 14" /></td>
<td>Traces*</td>
</tr>
<tr>
<td>15</td>
<td><img src="image5" alt="Structure 15" /></td>
<td>NO</td>
</tr>
<tr>
<td>16</td>
<td><img src="image6" alt="Structure 16" /></td>
<td>Traces</td>
</tr>
<tr>
<td>17</td>
<td><img src="image7" alt="Structure 17" /></td>
<td>Traces</td>
</tr>
<tr>
<td></td>
<td><img src="image1.png" alt="Chemical Structure" /></td>
<td>NO</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>18</td>
<td><img src="image2.png" alt="Chemical Structure" /></td>
<td>NO</td>
</tr>
</tbody>
</table>

*Traces amounts of a GSH adduct with an increased $m/z$ value of 303 was detected. This adduct is likely formed via aromatic epoxidation and subsequent loss of fluoride occurring on the molecule’s right hand side.*
FIG. 1

1

Valdecoxib

Oxacillin

Sulfamethoxazole
FIG. 2

A

B

C

GSH-adduct

Relative Abundance

Time (min)

Ox

Time (min)

P

Time (min)
FIG. 3

A

273
217

159

201

N

O

N

O

Cl

Cl

175

B

GSH adduct

506

552.0

m/z

m/z

Relative Abundance

100

90

80

70

60

50

40

30

20

10

0

100

150

200

250

300

350

400

500

550

600

650

133.1

159.0

201.1

217.1

230.0

333.3

357.2

377.0

506.2

606.1

This article has not been copyedited and formatted. The final version may differ from this version.

DMD Fast Forward. Published on August 20, 2012 as DOI: 10.1124/dmd.112.047431

Downloaded from dmjournals.aspetjournals.org on May 13, 2022.
FIG. 4

A

Cys

P

Relative Abundance

Time (min)

B

\[ \text{Chemical Structure} \]

C

\[ \text{NMR Spectra} \]

17

6

27(?)

8 & 12

9-11

*Cl-ring

DMSO-d5

Impurities

NH2 29

\[ \text{NMR Spectra} \]

\[ \text{Chemical Structure} \]
Fig. 6

This article has not been copyedited and formatted. The final version may differ from this version.
Supplemental data

Drug Metabolism and Disposition

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

Johan Bylund, Istvan Macsari, Yevgeni Besidski, Susanne Olofsson, Carl Petersson, Per I. Arvidsson and Tjerk Bueters

Synthesis isomeric phenyl 5'-methylisoxazole analogues

General

All materials were obtained from commercial supplier, unless otherwise noted, and used without further purification. Mass spectra were recorded either on Waters Alliance 2795 LC-MS (ZQ) with ESI, APCI and APPI in both positive and negative ionization mode or Agilent GC-MS with chemical ionization (CI, methane as reactant gas). For separation a capillary column was used, DB-5MS, (J&W Scientific). A linear temperature gradient was applied. Preparative chromatography was run on a Waters FractionLynx system with a MS-triggered fraction collection (ESI). Column; XBridge™ Prep C8 5 μm OBD™ 19 x 100 mm, with guard column; XTerra ® Prep MS C8 10 μm 19 x 10 mm Cartridge. A gradient from 100 % A (95 % 0.1 M ammonium acetate in MilliQ water and 5 % acetonitrile) to 100 % B (100 % acetonitrile) was
applied for LC-separation at flow rate 25 ml/min. Purity for final compounds was greater than 95% unless otherwise noted and measured on one of the following instruments. A) An Agilent HP1100 high performance liquid chromatography (HPLC) system with UV detections at 220, 254 and 290 nm. Phenomenex Gemini C18 3.0 mm x 50 mm, 3 μm, flow rate of 1.0 mL/min. A linear gradient was used starting at 100 % A (A: 10 mM ammonium acetate in 5 % acetonitrile) and ending at 100 % B (B: acetonitrile) after 3.5 minutes. B) A Waters Acquity UPLC system with UV detection at 254 nm was equipped with Waters ZQ mass spectrometer. Waters Acquity UPLC™ BEH C8 2.1 mm x 50 mm, 1.7 μm, flow rate 1.0 ml/min. A linear gradient ranging from 5 to 95% CH₃CN in H₂O with 0.01M ammonium acetate was used. The gradient was completed in 2min 15sec. The ZQ mass spectrometer was run with ESI in positive and negative mode. High resolution mass spectra (HRMS) were recorded on a Waters Synapt-G2 mass spectrometer (Waters MS Technologies, Manchester, UK) connected to an Acquity UPLC system with a PDA detector (Waters Corp., Milford, USA). All analyses were acquired using positive mode electrospray ionization (ESI+) in full scan and Leucine Encephalin (Sigma) was used as the lock mass (m/z 556.2771). Chromatographic separation was achieved with a 2.3 min gradient from 5-95% ACN (0.1% formic acid) over an ACQUITY UPLC BEH C18 1.7 μm, 2.1 x 50 mm column (Waters) maintained at 50°C and run at a flow rate of 0.4 mL/min. Microwave heating was performed in a Creator™ or Smith Synthesizer™ Single-mode microwave cavity producing continuous irradiation at 2450 MHz at the indicated temperature in the recommended microwave tubes. NMR spectra were recorded on a Varian 400 MHz, or on a Bruker 400 MHz and Bruker 500 MHz NMR spectrometer. The following reference signals were used: TMS δ 0.00, or the residual solvent signal of DMSO-d₆ δ 2.49, CD₃OD δ 3.31 or CDCl₃ δ 7.25 (unless otherwise indicated). Resonance multiplicities are denoted s, d, t, q, m and br for singlet, doublet, triplet,
quartet, multiplet and broad, respectively. Unless otherwise stated, chemical shifts are given in ppm with the solvent as internal standard. Column chromatography was performed using Merck Silica gel 60 (0.040-0.063 mm), or employing a Combi Flash® Companion™ system using RediSep™ normal-phase flash columns.

**Synthesis of compounds 18 and 19**

Compound 18 was prepared according to the scheme in Figure 1 as outlined below.

**Figure 1.** Reagents and conditions: (i) AgNO$_3$, NBS, acetone, rt; (ii) NaOCl, DCM, rt; (iii) PdCl$_2$.dppf, 2M K$_2$CO$_3$, DMF, MW, 130°C, 20 min; (iv) NaOH, THF, water, MW, 100°C, 8 min; (v) diphenylphosphoryl azide, TEA, dioxane, MW, 180°C, 5 min.
**5-(2,6-Dimethylphenyl)-3-methylisoxazole-4-carboxylic acid.**

*Step 1.* Silver nitrate (1.25 g, 7.5 mmol) followed by N-bromosuccinamide (14.45 g, 82.5 mmol) were added to a solution of ethylpropiolate (7.6 ml, 75 mmol) in acetone (125 mL). The resulting mixture was stirred for 2 hours at room temperature and filtered through a short bed of Celite. The filtrate was evaporated under reduced pressure. The crude residue was partitioned between DCM and water. The organic layer was separated, dried over magnesium sulfate, filtered and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography eluting with 2% EtOAc in hexane to give the desired product, 8.6 g (66%): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 4.21 (q, $J=7.16$ Hz, 2 H) 1.31 (t, $J=7.16$ Hz, 3 H).

*Step 2.* Sodium hypochlorite solution (93 ml, about 69.0 mmol) was added to a solution of 3-bromo-propynoic acid ethyl ester (2, 5.0g, 28.2 mmol) and acetaldoxime (2.5g, 42.3 mmol) in CH$_2$Cl$_2$ drop wise over 2.5 hours. The blue green solution was stirred for 30 minute and then quenched with water. The organic layer was separated, dried over magnesium sulfate, filtered and concentrated under reduced pressure to give the crude product, which was purified by silica gel column chromatography using with 10% ether in hexane as eluant. This offered the product as a regioisomeric mixture, 3.2 g (48%). The mixture was separated using preparative HPLC: LaPrep system; Column: Chiralpak AD; 50 x 300 mm, 10 μm; Mobile phase: 3 % ethanol / 97 % heptane; Flowrate: 120 mL/min, 350 mg/inj. The fractions were collected according to retention times yielding Isomer 1 as the first eluting fraction at 6.5 min and Isomer 2 at 8.5 min. The fraction were evaporated and handled separately. Ethyl 5-bromo-3-methyl-isoxazole-4-carboxylate: $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 4.35-4.29 (q, $J=7.16$ Hz, 2 H) 2.50 (s, 3 H)
1.34-1.41 (m, 3 H). Ethyl 4-bromo-3-methyl-isoxazole-5-carboxylate: $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 4.45 - 4.39 (q, $J$=7.16 Hz, 2 H) 2.36 (s, 2 H) 1.34 - 1.41 (m, 3 H).

**Step 3.** In a 20 mL microwave vial ethyl 5-bromo-3-methyl-isoxazole-4-carboxylate (0.5 g, 2.14 mmol), 2,6-dimethylphenylboronic acid (0.449 g, 2.99 mmol), PdCl$_2$(dppf)-CH$_2$Cl$_2$ adduct (0.174 g, 0.21 mmol) were mixed in DMF (10 mL) then 2M potassium carbonate solution (3.20 mL, 6.41 mmol) was added and set under N$_2$ atmosphere. The reaction mixture was heated in the microwave oven at 130 °C for 20 minutes. The crude was partitioned between water and DCM and the water phase was extracted twice with DCM. The combined organic phases were dried over anhydrous Na$_2$SO$_4$, filtered and evaporated. The crude was purified by column chromatography to yield ethyl 5-(2,6-dimethylphenyl)-3-methylisoxazole-4-carboxylate (0.260 g, 46.9 %) as a brown oil: $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ (ppm) 7.28 - 7.34 (m, 1 H) 7.15 (d, $J$=7.58 Hz, 2 H) 4.10 (q, $J$=7.16 Hz, 2 H) 2.53 (s, 3 H) 2.07 (s, 6 H) 1.03 (t, $J$=7.14 Hz, 3 H). GC-MS (CI) m/z 260 [M+1].

**Step 4.** Ethyl 5-(2,6-dimethylphenyl)-3-methylisoxazole-4-carboxylate (67 mg, 0.26 mmol) was stirred in water (0.3 mL) and THF (1.2 mL). Solid sodium hydroxide (20.67 mg, 0.52 mmol) was added and the mixture was stirred at rt for 40 hours. The reaction was slow, therefore microwave heating was applied for 8 minutes at 100°C. Thereafter the THF was removed in vacuo and the residue diluted with ethyl acetate and 2M NaOH solution before the phases were separated. The aqueous layer was treated with 4M HCl and extracted by EtOAc. The phases were separated and the aqueous layer was extracted again with EtOAc. The combined organic phase was dried over anhydrous Na$_2$SO$_4$ and concentrated. This crude was used without further purification. Colorless
solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 7.30 - 7.36 (m, 1 H) 7.14 (d, $J$=7.58 Hz, 2 H) 3.52 (s, 1 H) 2.58 (s, 3 H) 2.13 (s, 6 H). MS (ESI) $m/z$ 232 [M+1].

1-(5-(2,6-Dimethylphenyl)-3-methylisoxazol-4-yl)-3-((6-(tetrahydro-2H-pyran-4-yloxy)pyridin-3-yl)methyl)urea (18). In a 2 MW vial 5-(2,6-dimethylphenyl)-3-methylisoxazole-4-carboxylic acid (46 mg, 0.20 mmol), (6-(tetrahydro-2H-pyran-4-yloxy)pyridin-3-yl)methanamine (41.4 mg, 0.20 mmol), diphenylphosphoryl azide (54.7 mg, 0.20 mmol) and triethylamine (0.028 mL, 0.20 mmol) were mixed in dioxane (0.7 mL). The mixture was heated in the microwave oven at 180°C for 5 min. The crude was purified by prep-HPLC using 20-60% MeCN in NH$_4$OAc-buffer (0.1 M NH$_4$OAc in 95:5 water/MeCN), 13 min gradient. The product was obtained as colorless oil, 28 mg (32%): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 7.90 (br. s., 1 H) 7.31 - 7.36 (m, 1 H) 7.24 - 7.30 (m, 1 H) 7.07 (d, $J$=7.58 Hz, 2 H) 6.63 (d, $J$=8.34 Hz, 1 H) 5.83 (br. s., 1 H) 5.17 - 5.26 (m, 1 H) 4.68 (br. s., 1 H) 4.17 - 4.24 (m, 2 H) 3.95 - 4.03 (m, 2 H) 3.57 - 3.66 (m, 2 H) 3.49 (d, $J$=3.79 Hz, 1 H) 2.33 (s, 3 H) 2.09 (s, 6 H) 2.05 (br. s., 1 H) 1.72 - 1.84 (m, 2 H). MS (ESI) $m/z$ 437 [M+1].
Compound 19 was prepared according to scheme in Figure 2 as outlined below.

![Chemical diagram]

**Figure 2.** Reagents and conditions: (i) I₂, isopropylamine, rt; (ii) TEA, toluene, rt; (iii) AcOH, H₂NOH·HCl, 120°C; 1h (iv) NaOH, THF, MW, 100°C, 10 min; (v) diphenylphosphoryl azide, TEA, dioxane, MW, 180°C, 5 min.

5-(2,6-Dimethoxyphenyl)-3-methylisoxazole-4-carboxylic acid.

*Step 1.* Iodine (5.5 g, 21.67 mmol) was added to ethyl acetylacetone (20 mL, 157.83 mmol) then cooled down to 0°C. To this mixture isopropylamine (13.44 mL, 157.83 mmol) was slowly added. The mixture was allowed to stir at room temperature for 2 hours. Then the mixture was washed with water, sodium bisulfate solution and brine, dried over anhydrous sodium sulfate, filtered and concentrated. The crude was purified by silica gel column chromatography using 5 to
10% gradient ethyl acetate in heptane. (Z)-Ethyl 3-(isopropylamino)but-2-enoate (11 g, 41 %) was obtained as a liquid.

Step 2. Triethylamine (2.5 mL, 17.94 mmol) was added to a solution of (Z)-ethyl 3-(isopropylamino)but-2-enoate (3.2 g, 18.69 mmol) in toluene (15 mL). The reaction mixture was set under N₂ atmosphere and cooled down to 0°C. Then 2,6-dimethoxybenzoyl chloride (1.8 g, 8.97 mmol) in toluene (5 mL) was added to the mixture and allowed to warm up to room temperature then stirred for 16 hours. Thereafter the volatiles were removed in vacuo and the residue was purified by silica gel column chromatography using 10 to 20% gradient ethyl acetate in heptane. (Z)-Ethyl 2-(2,6-dimethoxybenzoyl)-3-(isopropylamino)but-2-enoate (1.6 g, 53 %) was obtained as solid.

Step 3. (Z)-Ethyl 2-(2,6-dimethoxybenzoyl)-3-(isopropylamino)but-2-enoate (1.8 g, 5.37 mmol) in acetic acid (30 mL) was treated with hydroxylamine hydrochloride (464 mg, 6.68 mmol) at room temperature then the mixture was stirred at 120°C for 1 hour. The reaction mixture was concentrated to 25% volume then diluted with water and extracted with ether. The organic phase was washed with saturated NaHCO₃ solution, brine then dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified by silica gel column chromatography using 20 to 30% gradient ethyl acetate in heptane. Ethyl 5-(2,6-dimethoxyphenyl)-3-methylisoxazole-4-carboxylate was obtained as solid 522 mg (35 %). 

\[ ^1H \text{ NMR (400 MHz, CDCl}_3\] \( \delta \text{ (ppm) 7.41 (t, } J=8.46 \text{ Hz, 1 H) 6.61 (d, } J=8.59 \text{ Hz, 2 H) 4.14 (q, } J=7.16 \text{ Hz, 2 H) 3.77 (s, 6 H) 2.54 (s, 3 H) 1.10 (t, } J=7.20 \text{ Hz, 3 H). MS (ESI) } m/\text{z} 292 \text{ [M+1].} \]

Step 4. Ethyl 5-(2,6-dimethoxyphenyl)-3-methylisoxazole-4-carboxylate (179 mg, 0.61 mmol) was stirred in water (0.8 mL) and THF (2.2 mL). Solid sodium hydroxide (49.2 mg, 1.23 mmol)
was added and the mixture was heated in a microwave (10 minutes at 100°C), however no reaction took place, so the temperature was increased up to 180°C (10 min, CAUTION!! The microwave vial does not tolerate this harsh condition for prolonged time!). THF was removed in vacuo and diluted by 2M NaOH solution and extracted by EtOAc. The aqueous layer was treated with 4M HCl and extracted by EtOAc. The phases were separated and the aqueous layer was extracted again with EtOAc. The combined organic phase was dried over anhydrous sodium sulfate and concentrated. White solid, 92 mg (57 %): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 7.43 (t, $J$=8.46 Hz, 1 H) 6.63 (d, $J$=8.46 Hz, 2 H) 3.78 (s, 6 H) 2.54 (s, 3 H). MS (ESI) $m/z$ 264 [M+1].

1-(5-(2,6-Dimethoxyphenyl)-3-methylisoxazol-4-yl)-3-((6-(tetrahydro-2H-pyran-4-yloxy)pyridin-3-yl)methyl)urea (19). The title compound was prepared as described for 18 using 5-(2,6-dimethoxyphenyl)-3-methylisoxazole-4-carboxylic acid (45mg, 0.17 mmol). The crude was purified by prep-HPLC using 15-55% MeCN in NH$_4$OAc-buffer (0.1 M NH$_4$OAc in 95:5 water/MeCN), 13 min gradient. The product was obtained as white solid, 22 mg (27%): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 7.90 (d, $J$=2.02 Hz, 1 H) 7.39 (t, $J$=8.46 Hz, 1 H) 7.32 - 7.37 (m, 1 H) 6.61 (d, $J$=8.59 Hz, 1 H) 6.57 (d, $J$=8.34 Hz, 2 H) 6.05 (br. s., 1 H) 5.16 - 5.24 (m, 1 H) 5.03 - 5.11 (m, 1 H) 4.23 (d, $J$=5.81 Hz, 2 H) 3.95 - 4.03 (m, 2 H) 3.69 (s, 6 H) 3.57 - 3.65 (m, 2 H) 2.29 (s, 3 H) 2.01 - 2.06 (m, 2 H) 1.72 - 1.83 (m, 2 H). MS (ESI) $m/z$ 469 [M+1].
NMR Analysis

Figure 3: The structure and numbering of compound 6.

Compound 6 (2mg) (Figure 3) was dissolved in 200 μl of methanol-d₄ and transferred to a 2.5 mm NMR-tube and 1D proton, selective 1D NOE, COSY, HSQC, and HMBC spectra were recorded. A sample temperature of 295 or 300 K was used. The same temperature was used for all experiments but it is unclear whether 295 or 300 K was used. The residual signal from the solvent was used as reference and set to 3.31 ppm for proton and 49.15 ppm for carbon. The proton spectrum could be assigned by the use of proton chemical shifts, intensities, J-couplings, and NOEs. In particular the NOE between CH₂ and the chlorinated ring assisted the assignments. The hetero nuclear experiments confirm the proton assignments. A proton spectrum with the assignments is shown in Figure 4 and 2D spectra are shown in Figure 5-7.
Figure 4: $^1$H NMR spectra with assignments for compound 6.
**Figure 5:** Gradient COSY for compound 6.
Figure 6: HSQC for compound 6.
Figure 7: Gradient COSY for compound 6.

Metabolite of 6:

The metabolite (unknown amount) was dissolved in approximately 100 μl DMSO-d₆ and transferred to a 2.5 mm NMR-tube prior to NMR analysis. Proton and TOCSY spectra were recorded at 318 K. The residual solvent signal was used as reference and set to 2.51 ppm. The proton spectrum of the metabolite was compared to the proton spectrum of 6. It is clearly seen that the CH₃ is no longer present in the metabolite and that a new CH₂ has been formed. The new
CH₂ has the splitting pattern typical of a CH₂ with non equivalent protons and no neigbouring (J-coupled) protons. This implies that the cystein group should be positioned on the newly formed CH₂ (the former CH₃ group). A predicted spectrum (ACD v9.06) of this metabolite gives a proton chemical shift for this CH₂ of 3.89 ppm while the actual chemical shift is measured to be 3.86 ppm. All signals from the Cys group could not be detected in the NMR spectrum. However, high resolution MS confirmed the accurate mass of a Cys adduct (not shown). Furthermore, the chemical shifts for the signals of proton 27 and 28 (Figure 8) are predicted (by ACD v9.06) to fall under the solvent signals. Assuming the Cys residue is conjugated at the sulfur atom, the NMR analysis of the metabolite confirms the structure shown in Figure 8. Figure 9 shows the assignment and Figure 10 shows the TOCSY spectrum.

**Figure 8:** The structure and numbering of the Cys adduct formed upon metabolism of 6.
Figure 9. Proton spectrum of the metabolite of 6 with assignments. Note the formation of the double doublet at 3.85 ppm with intensity corresponding to 2 protons and the lack of a singlet with intensity corresponding to 3 protons between 2-3 ppm, thus proving that the Cys residue is attached to carbon 6.
Figure 10: TOCSY for the Cys adduct of compound 6.