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

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Reactive metabolism of phenyl methyl-isoxazoles

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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; Na_{v}: 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 enimine 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 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 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 (Na\textsubscript{v} 1.7) (Macsari et al., 2011). Some of the most active compounds exhibited IC\textsubscript{50} values in the 10 nM range with promising selectivity towards Na\textsubscript{v} 1.5. Selectivity against Na\textsubscript{v} 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\textsubscript{v} 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 Sigma-Aldrich (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 Cl_{int} 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% CO_{2} 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. Cl_{int} values were obtained from disappearance curves of
the parent molecule (Floby et al., 2009). Compound 6 was included in all subsequent trapping studies as a positive control and reference.

**LC-MS analysis**

LC-MS analysis for Cl_int value estimations was performed with a Micromass Quattro Micro triple quadrupole as previously described (Floby et al., 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 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 (Figure 4).

The proton spectrum revealed that the CH₃ was replaced by a CH₂ 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 CH₂ 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 enamine intermediate (Table 2).

Trapping experiments showed that no GSH adducts were detected for compounds 11 and 12 that lacked the 5’-methyl group. NaV 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 \( \text{Cl}_{\text{int}} \) 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 Na\(_v\)1.7 antagonists exhibited potencies and selectivity toward Na\(_v\)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 \( \alpha \)-cyanocrolein followed by a condensation with formaldehyde to yield a reactive cyanoacrolein derivative (Yu et
al., 2011). 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 (Teffera 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 flucloxacinill, 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-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.

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 pattern 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

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Table 2. *In vitro* trapping experiments of modified Na,1.7 antagonist of the phenyl methyl-isoxazole series

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*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

Relative Abundance

Time (min)

Time (min)

Time (min)

PP

GSH-adduct
FIG. 3

A

B

GSH adduct

m/z

Relative Abundance

m/z

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FIG. 5

The figure shows a chemical reaction process involving various molecules. The reaction pathway includes the following steps:

1. CYP (cytochrome P450) catalyzes the reaction.
2. O (oxygen) reacts with the molecule.
3. H₂O (water) is produced as a byproduct.
4. H₂ (hydrogen) reacts with the molecule.
5. GSH (glutathione) is involved in the reaction.
6. The final product is shown at the bottom of the diagram.
FIG. 6

A

GSH-adduct +305

GSH-adducts +321 and +337

Time (min)

B

GSH-adduct +305

GSH-adduct +321

Time (min)

C

GSH-adduct +305

Time (min)