Short Communication

Aromatic Substitution Reaction of 2-Chloropyridines Catalyzed by Microsomal Glutathione S-Transferase 1

Received March 24, 2009; accepted June 17, 2009

ABSTRACT:

We investigated the substitution reaction of a series of 2-chloropyridine derivatives catalyzed by rat liver microsomal glutathione S-transferase 1. Various 2-chloropyridine derivatives were metabolized to the corresponding substituted glutathione conjugates via displacement of halogen atom with glutathione. The reaction was affected by the electron-withdrawing strength and position of the substituents. Molecular orbital calculations on the change in Gibbs free energy between the initial and transition states verified the presence of a Meisenheimer complex and its influence on the reaction rate.

Glutathione S-transferase (GST) catalyzes the conjugation between glutathione (GSH) and various endogenous or xenobiotic electrophilic compounds. It is interesting to note that such GST-catalyzed reactions have also included several nucleophilic aromatic substitutions with GSH (Habig et al., 1974; Clark and Sinclair, 1988; Armstrong, 1991, 1997; Patkovsky et al., 2006; Teffera et al., 2008), in which halogenated nitrobenzene derivatives have been used as substrates to generate GSH adducts via displacement of halogen atom with GSH. Three major families of proteins that are widely distributed in nature exhibit GST activity. Two of these, the cytosolic and mitochondrial GST, comprise soluble enzymes. The third family comprises membrane-bound microsomal GST. The chemical mechanism of soluble GST has been well studied. The enzyme initially ionizes the sulfhydryl group of GSH to form a strong nucleophilic thiolate anion (GSH-) that spontaneously attacks the electrophilic center of the substrates forming a negatively charged σ-complex or Meisenheimer complex as an intermediate (Habig et al., 1974; Keen et al., 1976; Clark and Sinclair, 1988; Armstrong, 1991, 1997; Chen et al., 2004; Patkovsky et al., 2006). Accordingly, the electrophilicity of the substrates, as determined by the nature and position of the substituent groups, has been shown to affect the catalytic activity of GST toward GSH conjugation. On the other hand, Morgenstern et al. (2001) revealed that an important aspect of the catalytic mechanism of microsomal GST 1 (MGST1) is the activation of the thiol of bound GSH while the rate-determining step shifts from thiolate formation to the chemical reaction as the chemical reactivity of the electrophile becomes lower. Herein, we describe a substitution reaction between GSH and various 2-chloropyridine derivatives catalyzed by rat liver MGST1 to form the corresponding 2-glutathionyl-pyridine derivatives. Furthermore, investigations were carried out to study factors that affect the reactivity of the 2-chloropyridine derivatives toward GSH.

Materials and Methods

Chemicals. GSH, glutathione sulfonic acid, 1-chloro-3,4-dinitrobenzene (CDNB), 2,3-dichloropyridine, 3-amino-2-chloropyridine, 2-chloro-3-methylpyridine, and potassium phosphate buffer were purchased from Sigma-Aldrich (St. Louis, MO). 2-Chloro-4-nitropyridine, 2-chloro-5-nitropropyridine, 2-chloro-3-hydroxypyridine, and 2-chloro-3-trifluoropyridine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 2-Chloro-3-methoxy-pyridine and 2-chloro-3-nitro-pyridine were purchased from Lancaster (Panorama City, CA). Magnesium chloride and acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pooled SD rat liver microsomes were obtained from XenoTech, LLC (Lenexa, KS).

Incubation Conditions. Incubation mixtures consisted of 0.1 M potassium phosphate buffer (pH 7.4), 3 mM magnesium chloride, 1 mg/ml rat liver microsomes, and 5 mM GSH. The incubation mixtures were preincubated for 10 min. Incubation was initiated by the addition of the compounds and was conducted at 37°C with continuous shaking. The reaction mixture with 2-chloro-3-nitro-pyridine (100 μM) was incubated for 60 min. Inhibitory effect of glutathione sulfonic acid (20 mM) on the substitution activity of liver MGST1 was determined. To investigate the position effects of the nitro group, reaction rates (nmol/min/mg) of incubations involving various 2-chloro-pyridine and nitrobenzene derivatives (1 mM) were determined over varying durations (1–40 min). To investigate the substituent effects, 30-min incubations were carried out using reaction mixtures with the concentration of the derivatives at 50 μM. The enzymatic reactions were terminated by the addition of an equal volume of acetonitrile/methanol (2:1, v/v). Upon termination, the reaction mixture was subjected to centrifugation (4°C, 13,800 g, 10 min), and the resulting supernatant was analyzed using liquid chromatography/mass spectrometry or liquid chromatography/UV.

Analytical Procedure. Liquid chromatography/UV analyses were conducted using an Alliance 2790 Separations Module (Waters, Milford, MA) equipped with UV detectors (2478 Dual μ Absorbance Detector and Photo diode Array Detector; Waters). Chromatographic separations were performed on a Cadenza 3 μm C18, 2 × 100-mm column (Imtak, Kyoto, Japan) at a flow rate of 0.3 ml/min under gradient conditions (13 min). The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Solvent B was linearly increased from 5 to 95% over 5 min, maintained at 95% for 4 min, then decreased to 5% for 4 min (equilibration).

ABBREVIATIONS: GST, glutathione S-transferase; GSH, glutathione; MGST1, microsomal GST 1; CDNB, 1-chloro-3,4-dinitrobenzene; QSAR, quantitative structure-activity relationship.
Chromatographic separations were performed on a Cadenza 3. The GSH adduct exhibited a protonated molecular ion at m/z 430, corresponding to the elimination of pyroglutamate and glycine residues of GSH, respectively.

**GST Dependence of 2-Glutathionyl-3-nitropyridine Formation.** The formation of the GSH adduct from 2-chloro-3-nitropyridine requires liver microsomes, and the reaction was inhibited by 72% by 20 mM glutathione sulfonic acid, a competitive inhibitor of GSTs (Sun and Morgenstern, 1997), suggesting that these results demonstrate the catalytic activity of MGST1. In the absence of liver microsomes, the reaction in buffer (pH 7.4, 37°C) afforded a small amount of the GSH adduct (5% of the total formation compared with the sample incubated with rat liver microsomes), suggesting that 2-chloro-3-nitropyridine can nonenzymatically react with GSH to some extent.

**Effects of the Nitro Group.** To investigate the effects of a nitro group and the pyridine structure for the substitution reactions, the substitution reactions were carried out using 2-chloropyridine, 1-chloro-2-nitrobenzene, CDNB, 2-chloro-3-nitropyridine, 2-chloro-4-nitropyridine, and 2-chloro-5-nitropyridine as substrates. As shown in Table 1, the lack of reaction for 2-chloropyridine and the weak reaction for 1-chloro-2-nitrobenzene (6.8 nmol/min/mg) suggested the importance of the nitro group and the pyridine structure, respectively. The highest activity was observed for 2-chloro-3-nitropyridine (236 nmol/min/mg) and was comparable with that of CDNB (306 nmol/min/mg), a well known substrate for GST. The activities of 2-chloro-4-nitropyridine (11.5 nmol/min/mg) and 2-chloro-5-nitropyridine (137 nmol/min/mg) suggest that the reactivity of the 2-chloro-nitropyridine derivatives depends on the position of the nitro group, which exerts a strong electron-withdrawing effect at its ortho- and para-positions. Furthermore, pyridine itself possesses an electron-deficient nature at the C2-carbon due to the resonance of a pyridinium cation, in which a positive charge is distributed onto C2. Nucleophilic aromatic substitution reactions with GSH, therefore, should be favorable at the 2-position (reaction center) of 3- and 5-nitropyridines, which are highly electron-deficient. Substituent effects on the GSH substitution reaction were investigated using various 3-substituted 2-chloropyridine derivatives that possess electron-withdrawing groups such as nitro (NO2), trifluoro (CF3), and chloro (Cl), or electron-donating groups such as methyl (Me), methoxy (MeO), amino (NH2), and hydroxy (OH) as substrates. As shown in Table 2, the activities of
TABLE 1
Activity of substitution reaction for 2-chloropyridine/chlorobenzene derivatives in rat liver microsomes fortified with GSH

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity (nmol/min/mg)</th>
<th>ΔG‡ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Chloro-3,4-dinitrobenzene</td>
<td>306 ± 12</td>
<td>12.1</td>
</tr>
<tr>
<td>2-Chloro-3-nitropyridine</td>
<td>236 ± 8</td>
<td>19.6</td>
</tr>
<tr>
<td>2-Chloro-5-nitropyridine</td>
<td>137 ± 5</td>
<td>16.0</td>
</tr>
<tr>
<td>2-Chloro-4-nitropyridine</td>
<td>11.5 ± 1.0</td>
<td>24.8</td>
</tr>
<tr>
<td>1-Chloro-2-nitrobenzene</td>
<td>6.8 ± 1.1</td>
<td>24.6</td>
</tr>
<tr>
<td>2-Chloropyridine</td>
<td>&lt;2</td>
<td>39.6</td>
</tr>
</tbody>
</table>

(2001) also reported that, under the condition in which GSH and enzyme were mixed in advance for presteady-state kinetics analysis, the rate constant (kₐ) for the chemical reaction between GS⁻ and substrates is linearly related to the chemical reactivity of the substrates as is evident in the Hammett plot. As shown in Tables 1 and 2, molecular orbital calculations of various 2-chloropyridine derivatives indicate an inverse correlation between reaction rates and changes in the Gibbs free energy between the initial and transition states, ΔG‡. These results strongly support the putative reaction mechanism (Fig. 2) featuring the Meisenheimer complex.

Many studies on quantitative structure-activity relationships (QSARs) for GSTs have been reported to date. For example, Keen et al. (1976) first presented Hammett plots of the catalytic constants of GST for the GSH conjugation reaction. A more detailed analysis for cytosolic GST 4-4 using multiple computer-calculated molecular parameters was conducted by Van der Aar et al. (1996, 1997) and showed good correlations between the GSH conjugation of 4-substituted 1-chloro-2-nitrobenzenes and their Hammett constants, although lower correlation coefficients were obtained for 2-substituted 1-chloro-4-nitrobenzenes. Rietjens et al. (1995) used semiempirical computer-calculated molecular parameters based on frontier orbital theory and on transition state calculations for describing QSAR for the GSH reaction of fluoronitrobenzenes catalyzed by cytosolic GST to elucidate the pathway via Meisenheimer complex. In this study, linear correlations were observed between ln V_max and E_homo (energy of the lowest unoccupied molecular orbital) and between ln V_max and ΔΔHF (relative heat of formation for formation of the respective Meisenheimer complex). The latter parameter was based upon the calculations representative for relative changes in the transition state and the intermediates of the reaction and seems to be close to ΔG‡ in the present study, which might provide an alternative approach for characterizing QSAR for the conversion rate of a series of substrates by GST.

In conclusion, we have shown that nucleophilic aromatic substitution reaction of 2-halogenated pyridine derivatives, which is a unique metabolic pathway catalyzed by MGST1, occurs in liver microsomes. The reaction was affected by both the position, relative to C2 (reaction center), and the electron-withdrawing strength of the substituents. Molecular orbital calculations suggested that the key step of these reactions involves the formation of a Meisenheimer complex, as previously proposed. It is important to note that 2-halogenated pyridine derivatives, which are often seen in drug discovery, can acquire reactivity toward GSH, depending on the electron-withdrawing properties of the substituents. Such reactivity may affect the reactivity between nucleophilic macromolecules and drug candidates, and it may potentially lead to adverse effects involving irreversible binding to macromolecules. In fact, we observed the significant covariant binding to proteins after microsomal incubation with some compounds containing 2-halogenated pyridine structure at drug discovery phase (detailed results will be reported in a separate publication). Therefore, our findings in the present study offer important insight into structural modifications at the lead optimization stage to circum
vent adverse chemical reactivity. Further studies on the versatility of this metabolic pathway are in progress using structurally diverse compounds.

Department of BY Preclinical DMPK (K.I., T.O., Y.I., M.C.) and Department of Chemistry (K.M., T.S.), Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba, Ibaraki, Japan

References


Address correspondence to: Tomoyuki Ohe, Department of BY Preclinical DMPK, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Okubo 3, Tsukuba, Ibaraki 300-2611, Japan. E-mail: tomoyuki_ohe@merck.com

KAZUKO INOUE  
TOMOYUKI OHE  
KENICHI MORI  
TAKESHI SAGARA  
YASUYUKI ISHI  
MASATO CHIBA