Contribution of Artifacts to N-Methylated Piperazine Cyanide Adduct Formation In Vitro from N-Alkyl Piperazine Analogs

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ABSTRACT

In the liver microsome cyanide (CN)-trapping assays, piperazine-containing compounds formed significant N-methyl piperazine CN adducts. Two pathways for the N-methyl piperazine CN adduct formation were proposed: 1) The α-carbon in the N-methyl piperazine is oxidized to form a reactive iminium ion that can react with cyanide ion; 2) N-dealkylation occurs followed by condensation with formaldehyde and dehydration to produce N-methylene piperazine iminium ion, which then reacts with cyanide ion to form the N-methyl CN adduct. The CN adduct from the second pathway was believed to be an artifact or metabonate. In the present study, a group of 4′-N-alkyl piperazines and 4′-N-[13C]methyl-labeled piperazines were used to determine which pathway was predominant. Following microsomal incubations in the presence of cyanide ions, a significant percentage of 4′-N-[13C]methyl group in the CN adduct was replaced by an unlabeled natural methyl group, suggesting that the second pathway was predominant. For 4′-N-alkyl piperazine, the level of 4′-N-methyl piperazine CN adduct formation was limited by the extent of prior 4′-N-dealkylation. In a separate study, when 4′-NH-piperazine were incubated with potassium cyanide and [13C]-labeled formaldehyde, 4′-N-[13C]methyl piperazine CN adduct was formed without NADPH or liver microsome suggesting a direct Mannich reaction is involved. However, when [13C]-labeled methanol or potassium carbonate was used as the one-carbon donor, 4′-N-[13C]methyl piperazine CN adduct was not detected without liver microsome or NADPH present. The biologic and toxicological implications of bioactivation via the second pathway necessitate further investigation because these one-carbon donors for the formation of reactive iminium ions could be endogenous and readily available in vivo.

Introduction

Drug-induced toxicity is one of the major reasons for withdrawal of marketed drugs (Kaplowitz, 2005). Investigation of the underlying mechanisms for adverse drug reactions (ADRs) is among the major efforts of pharmaceutical companies, along with managing risks associated with acute toxicities (Smith and Obach, 2009). Drugs that are metabolized to generate electrophilic reactive intermediates, which can form covalent adducts with macromolecules, carry an increased risk of ADRs or idiosyncratic toxicity (Waring and Anderson, 2005; Waring and Ulrich, 2007) and drug-drug interactions caused by mechanism-based inactivation (MBI) of cytochrome P450 enzymes (Orr et al., 2012). Glutathione, potassium cyanide (KCN), methoxyamine, and semicarbazide have become widely used as trapping agents to evaluate the metabolic activation potential of drug molecules (Evans et al., 2004; Gorrod and Aislaitner, 1994). These alicyclic amine, such as nicotine and prolintane, which form hard electrophilic iminium ions, are highly reactive with cyanide, suggesting their potential to react with macromolecules (Murphy, 1973). Other alicyclic amines, such as nefazodone, indinavir, and prochlorperazine, have also been shown to form iminium ions that react with cyanide (Gorrod and Aislaitner, 1994).

In CN-trapping assays performed on secondary alicyclic amines, such as the nefazodone N-dealkylation product, significant CN adduct was found with an addition of 14 Da in the molecule (Argoti et al., 2005; Bauman et al., 2008). In our CN-trapping screening on N-dealkylated alicyclic compounds, significant CN adducts with an additional 14 Da were also observed and confirmed as the addition of a methylene group (unpublished data). It has been suggested that this type of N-methylated CN adduct from the secondary alicyclic amines was an experimental artifact, or “metabonate,” from an in vitro microsomal incubation system (Gorrod and Sai, 1997; Li et al., 2006; Rousu and Tolonen, 2011; Barbara et al., 2012). In the case of N-methyl piperazines, there are two possible pathways for the formation of N-methyl piperazine CN adducts (Fig. 1). In pathway 1, the iminium ion is formed via α-carbon oxidation, which could have potential toxicological implications (Sayre et al., 1997) and cause MBI of the cytochrome P450 enzymes (Orr et al., 2012). In pathway 2, N-dealkylation results in the formation of a secondary alicyclic amine, which can react with formaldehyde and eventually lead to the formation of an iminium ion. The CN adduct from pathway 2 has been considered the artifact from the in vitro system and irrelevant to in

ABBRVIATIONS: ADR, adverse drug reaction; amu, atomic mass unit; CE, collision energy; CN, cyanide; HCN, hydrogen cyanide; HLM, human liver microsomes; KCN, potassium cyanide; LC/MS, liquid chromatography coupled with mass spectrometry; MBI, mechanism-based inactivation; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio; MS, mass spectrometry; NL, neutral loss; NL-27, neutral loss of 27 amu; RLM, rat liver microsomes; UPLC, ultraperformance liquid chromatography.
vivo (Gorrod and Sai, 1997; Rousu and Tolonen, 2011; Barbara et al., 2012). In the study reported here, we have evaluated the mechanism of CN adduct formation in several piperazine-containing compounds (Fig. 2) with secondary and tertiary alicyclic amines. Piperazine compounds with [13C]-labeled 4'-N-methyl group were used to quantify the contribution of each pathway to the formation of 4'-N-methyl piperazine CN adduct in liver microsomal incubations. Furthermore, by identifying the one-carbon sources for the formation of the 4'-N-methyl piperazine iminium ion, we have demonstrated the relevance of pathway 2 of CN adduct formation to the in vivo system and, thereby, to the potential ADR and MBI liabilities.

Materials and Methods

The 4'-N-alkyl piperazine compounds tetrahydronaphthalenes (III, IV), chromans (VII and X–XIV), and the 4'-N-desmethyl piperazine compounds (I, VI, and IX) were synthesized by AstraZeneca (Wilmington, DE). [13C]Paraformaldehyde was purchased from Cambridge Isotope Laboratory (Andover, MA). High-performance liquid chromatography (LC) water, methanol, potassium carbonate, and acetonitrile were purchased from Thermo Fisher Scientific Co. (Pittsburg, PA). Potassium phosphate, dimethylsulfoxide, LC coupled with mass spectrometry (LC/MS)-grade formic acid, ammonium formate, NADPH, magnesium chloride, sodium cyanoborohydride, triethylamine, bromoacetanitrile, KCN, potassium [13C]carbonate, [13C]methanol, [13C]formaldehyde, and other chemical reagents used were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLMs) were purchased from BD Gentest (San Jose, CA). Rat liver microsomes (RLMs) were purchased from CellzDirect (Austin, TX).

Synthesis of Cyanide Adduct Standard (II) and 4'-N-[13C]Methyl Piperazines (V and VIII)

Cyanomethyl piperazine analog (II) was prepared by alkylation of the des-methyl precursor with bromoacetanitrile and potassium carbonate in dimethylsulfoxide. The [13C]-labeled adducts (V and VIII) were prepared by the reductive amination of the des-methyl precursor with [13C]paraformaldehyde, sodium cyanoborohydride, and triethylamine in methanol. The structures were confirmed by nuclear magnetic resonance, and the purity was determined by LC/ultraviolet. The purity of the [13C]-labeled compounds was greater than 98%.

Microsomal Incubation

Formation of the CN adduct was measured in HLM and RLM incubations. Unless specified, the 1-ml incubation media contained 10 μM test compound.
1 mg/ml of microsomal protein, 5 mM magnesium chloride, 5 mM KCN, and 1 mM NADPH in 100 mM phosphate buffer, pH 7.4. After preincubation at 37°C for 3 minutes, the reaction was initiated by the addition of the test compound. Equal volumes of cold acetonitrile were added to terminate the reaction at 30 minutes. After removal of denatured protein by centrifugation, the supernatant was analyzed by LC coupled with mass spectrometry.

To investigate the carbon source for N-methylated piperazine CN-adduct, 10 μM 4′-N-desmethyl piperazine compound (I or IX) was incubated with HLM in a phosphate buffer (pH 7.4) in the presence of KCN as described, with either 5 mM [13C]-labeled potassium carbonate, 1% of methanol (v/v), or 1% of formaldehyde (v/v) included in the incubation. The reaction was initiated by the addition of NADPH. The control incubations that contained all components except KCN were performed. To determine the contribution of enzymes and NADPH to the formation of CN adducts, compound I or IX was also incubated in a solution containing all components except liver microsome or NADPH. In a separate study, compound I was incubated in HLM in the presence of 5 mM [13C]-labeled potassium carbonate, 5 mM KCN with the addition of semi-carbazide ranging from 0.3 to 3 mM to determine its impact on the formation of CN adduct by removing aldehyde intermediate formed during the incubation.

**LC/MS Analysis**

The CN adducts formed in microsomal incubation were analyzed by ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA) coupled with API 4000 QTRAP (Applied Biosystems, Foster City, CA) or LTQ Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA). A 2.1 × 100-mm BEH C18 column with 1.7-μm particle size (Waters) was used for the UPLC separation. Eluting solvents consisted of 0.1% formic acid and 5% acetonitrile in LC/MS grade water (A) and 0.1% formic acid in acetonitrile (B). The elution gradient, at flow rate of 0.2 ml/min, started with 0% B, linearly increased to 20% B in 4 minutes, and then linearly increased to 80% B in 3 minutes, to 95%B in another 2 minutes, and held at 95% B for additional 2 minutes. The column was then equilibrated by washing with 100% A for 3 minutes.

**API 4000 QTRAP Analysis.** The UPLC eluants were ionized under positive electrospray ionization mode with curtain gas set at 25, collision gas at 10, ion spray voltage at 4500 V, temperature at 450°C, and the sheath gas flow rates at 60 arbitrary units. The Orbitrap Fourier transform mass spectrometer resolution was set at 15,000 with a scan range at 50–600 m/z. Data-dependent MS2 and MS3 fragmentation were obtained in the linear ion trap where the normalized CE was set at 35 with 0.23 activation Q and 30-millisecond activation time.

**Data Processing**

Accurate mass data acquisition, process, and theoretical isotope spectra simulation were conducted with Xcalibur 2.1 (Thermo Scientific). Unit resolution mass data and NL-27 chromatograms were generated with Analyst version 1.4.2 (AB Sciex, Foster City, CA). The natural abundance ratio of 13C:12C is 1.1:98.9. Theoretical 13C relative abundance is determined by multiplying 1.1 by the spectrometry (MS/MS) fragmentation for ions exceeding 10,000 counts per second from the enhanced MS scan or for ions exceeding 1000 counts per second from the scan for neutral loss (NL) of 27 (NL-27) atomic mass units (amu) with a dwell time of 30 milliseconds. The collision energy (CE) for the NL and enhanced product ionization scans was set at 25 and 45 V, respectively.

**LTQ Orbitrap Analysis.** The CN adduct structures were also analyzed by Orbitrap mass spectrometer under positive electrospray ionization mode with source voltage at 4 kV, capillary temperature at 275°C, and the sheath gas flow rates at 60 arbitrary units. The Orbitrap Fourier transform mass spectrometer resolution was set at 15,000 with a scan range at 50–600 m/z. Data-dependent MS2 and MS3 fragmentation were obtained in the linear ion trap where the normalized CE was set at 35 with 0.23 activation Q and 30-millisecond activation time.

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**Fig. 3.** NL-27 ion chromatogram monitoring m/z 446 ion (4′-N-desmethyl piperazine CN adduct, early small peak) and m/z 460 ion (4′-N-methyl piperazine CN adduct, major peak) in HLM (A) and RLM (B) incubations of compound I in the presence of KCN.

**Fig. 4.** NL-27 ion chromatogram monitoring m/z 460 (blue for [12C]) and 461 (red for [13C]) ions of the synthetic standard (II).

**Fig. 5.** NL-27 ion chromatogram monitoring m/z 460 ions (4′-N-methyl piperazine CN adducts) in HLM (A) and RLM (B) incubations of compound IV in the presence of KCN.
number of carbon atoms in the molecule. For example, for a molecule with 26 carbon atoms, the $^{13}$C natural abundance in MS ion counts would be 28.6% of the $^{12}$C ion counts. For the $[^{13}$C]-labeled compounds, only a pure $^{13}$C peak would be observed, and the changes in $^{13}$C/$^{12}$C ratio of the CN adduct with respect to the natural $^{13}$C abundance of the reference cyanide adduct would indicate the extent of contribution from pathway 2 (Fig. 1). When the contribution of pathway 1 is defined as $\alpha$ for the fraction of labeled $^{13}$C remaining in the molecule and the contribution of pathway 2 as $(1 - \alpha)$ for the $^{12}$C added to the molecule, the total $^{13}$C in the molecule would be $\alpha + \beta \times (1 - \alpha)$, where $\beta$ is the natural $^{13}$C abundance gained from pathway 2. If $A$ is defined as the total $^{13}$C/$^{12}$C ratio, then $A = \alpha + \beta \times (1 - \alpha)/(1 - \alpha)$, from which, after rearranging the equation, the contribution of pathway 1 can be obtained, $\alpha = (A - \beta)/(1 + A - \beta)$, where $A$ can be measured by mass spectrometer ion peak intensities and $\beta$ derived from the natural $^{13}$C abundance of $^{12}$C in pathway 2.

**Results**

**CN Adduct Formation and Structure Confirmation**

**Tetrahydroanaphelene Compounds.** When $^{4}'$-N-desmethyl piperazine (I) was incubated in HLM or RLM with trapping agent KCN, a major CN adduct peak at retention time of 8.7 minutes was detected in LC/MS analysis monitoring NL-27 ions (Fig. 3). This CN adduct had a molecular ion at $m/z$ 460.2716 ($C_{27}H_{34}O_2N_5$, $\Delta = 2.0$ ppm), which is consistent with the addition of a methyl and a CN group. This CN adduct peak matched the synthetic standard (II) peak in both retention time and MS characteristics (Fig. 4). When $^{4}'$-N-methyl piperazine (IV) was incubated in HLM or RLM with KCN, three $^{4}'$-N-methyl piperazine CN adduct peaks were detected in the NL-27 ion chromatograms, with the parent molecular ion at $m/z$ 460 (Fig. 5).

Structural assignments of these three CN adducts generated from IV were based on the MS/MS fragmentation patterns and the chromatographic peak retention time in comparison with the synthetic standard (II). The $m/z$ 433 fragment ions as the result of the NL of hydrogen cyanide (HCN) group from the molecular ion of the CN adducts at $m/z$ 460 and the benzoic-morpholine ions ($m/z$ 190 or 188) were the signature

**Fig. 6.** MS$^2$ fragmentation patterns of $^{4}'$-N-methyl piperazine CN-adducts of compound (IV). (A) Synthetic standard (2). (B–D) CN adduct peaks 1–3, respectively (presented in Fig. 5).

**Fig. 7.** NL-27 ion chromatogram monitoring $m/z$ 460 (blue for $^{12}$C) and $m/z$ 461 (red for $^{13}$C) ions of $^{4}'$-N-methyl piperazine CN-adducts in HLM (A) and RLM (B) incubations of compound V in the presence of KCN.
fragments of these CN adducts (Fig. 6). When the CN group was in the piperazine moiety, the intact benzoic-morpholine ion fragment \((m/z 190)\) was observed (Fig. 6, A, C, and D). Peak 1 generated a fragment at \(m/z 188\), consistent with the benzoic-morpholine fragment after an NL of HCN group from the morpholine ring (Fig. 6B); therefore, the CN adduct group was assigned to morpholine. The retention time of peak 3 (Fig. 5) and its fragmentation patterns (Fig. 6D) matched those of the synthetic standard II (Figs. 4 and 6A, respectively), confirming that in peak 3, the CN adduct was in the 4\(\,^9\)-N-methyl group. Peak 2 generated a benzoic-morpholine fragment at \(m/z 190\) (Fig. 6C), but the retention time did not match standard II, indicating that its CN adduct is unlikely at the 4\(\,^9\)-N-methyl group; therefore, the only other place left in piperazine to assign the CN adduct would be at the 2\(\,^9\) or 3\(\,^9\)-position.

When 4\(\,^9\)-N-[\(^{13}\)C]methyl piperazine (V) was incubated with (either rat or human) liver microsomes, the same peaks 1, 2, and 3 were formed (Fig. 7). Since the CN adduct in peak 1 was in the morpholine ring, the 4\(\,^9\)-N-[\(^{13}\)C]methyl piperazine was intact in the molecule, and there was no loss of [\(^{13}\)C]-label in the peak. In peak 2, [\(^{13}\)C]methyl group was also retained demonstrating that the methyl piperazine CN adducts in peak 2 was not preceded by 4\(\,^9\)-N-demethylation; rather, it was through \(\alpha\)-oxidation at 2\(\,^9\) or 3\(\,^9\) position (pathway 1). However, in peak 3, a significant amount of 4\(\,^9\)-N-[\(^{13}\)C]methyl group was replaced by natural 12C-methyl group (Fig. 7), indicating that most methyl piperazine CN adducts in peak 3 were formed after the 4\(\,^9\)-N-demethylation (pathway 2) as illustrated in Fig. 8.

**Chroman Compounds.** As in the tetrahydronapthalene compounds under the same incubation conditions, 4\(\,^9\)-N-desmethyl piperazine (VI) also generated a major 4\(\,^9\)-N-methyl piperazine CN-adduct (Fig. 9). Accurate mass analysis of this CN adduct showed a molecular ion at \(m/z\) 480.2424 (C\(_{26}\)H\(_{31}\)O\(_3\)N\(_5\)F, \(\Delta = 2.7\) ppm), consistent with the

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**Fig. 8.** Proposed pathway of tetrahydronapthalene compound (V) 4\(\,^9\)-N-methyl piperazine CN adduct formation in liver microsomal incubations in the presence of KCN.

**Fig. 9.** NL-27 ion chromatogram monitoring \(m/z\) 466 ion (4\(\,^9\)-N-desmethyl piperazine CN adduct, early small peak) and \(m/z\) 480 ion (4\(\,^9\)-N-methyl piperazine CN adduct, major peak) in HLM (A) and RLM (B) incubations of compound VI in the presence of KCN.

**Fig. 10.** NL-27 ion chromatogram monitoring \(m/z\) 480 ions (4\(\,^9\)-N-methyl piperazine CN adducts) in HLM (A) and RLM (B) incubations of compound VII in the presence of KCN.
addition of methyl and CN group to compound VI. Three major and one minor methyl piperazine CN-adduct peaks were observed in microsomal incubations of compound VII with KCN (Fig. 10), and peak 3 (RT 8.8 minutes) matched the 4'-N-methyl piperazine CN-adduct peak from VI in Fig. 9. The fragmentation pattern of the parent compound VII revealed that the fragment ion at m/z 277 was consistent with the chroman-8-(4'-N-methylpiperazine)-2-carbonyl moiety (Fig. 11A), which could be used as signature fragment ion to locate the sites of CN adducts. Fragmentation of 4'-N-methyl CN-adduct ion at m/z 480 from peaks 1, 2, and 3 (Fig. 10) generated a product ion at m/z 453 as the result of an NL of 27 (HCN). Further fragmentation of the ion at m/z 453 generated a product ion at m/z 275, which is consistent with the chroman-8-(4'-N-methylpiperazine)-2-carbonyl moiety upon an NL of HCN (Fig. 11, B–D), suggesting that the CN adduct was in the piperazine moiety for peaks 1–3. Because of its low quantity, the minor methyl piperazine CN adduct peak shown in Fig. 10 did not trigger fragmentation and was not evaluated further. The MS/MS fragmentation pattern of the 4'-N-methyl piperazine CN-adduct (major peak in Fig. 9) of N-desmethyl piperazine (VI) was similar to those of the 4'-N-methyl piperazine CN-adducts (peaks 1–3) generated from VII (unpublished data). Similar results were observed for compounds IX and X (unpublished data). When 4'-N-[13C]methyl piperazine compound (VIII) was incubated in liver microsomes with KCN, a significant [13C]methyl group was replaced by natural 4'-N-[12C]methyl group in CN adduct peak 3, whereas in peaks 1 and 2, the 4'-N-[13C]methyl group was retained in the CN adducts (Fig. 12), confirming that the CN adduct in peak 3 was associated with 4'-N in the piperazine and that most CN adduct in peak 3 was formed via pathway 2 as illustrated in Fig. 13.

**Contribution of Pathway 2 in the Formation of 4'-N-Methyl Piperazine CN Adduct**

The contributions of pathways 1 and 2 to the formation of CN adducts associated with 4'-N-methyl piperazine were estimated by measuring the $^{13}$C/$^{12}$C ratio change after the incubation of [13C]methyl labeled compounds (V and VIII) with liver microsomes in the presence of KCN and by analyzing the relationship between the extent of 4'-N-dealkylation and 4'-N-methyl piperazine CN adduct formation. After microsomal incubations of the 4'-N-[13C]methyl piperazines (V or VIII) with KCN, a fraction of CN adduct in peak 3 retained 4'-N-[13C]methyl group, suggesting bioactivation via pathway 1 (Figs. 7 and 12). However, a significant proportion of 4'-N-[13C]methyl group was replaced with natural 4'-N-[12C]methyl group in the CN adduct peak 3, indicating a significant contribution of pathway 2 (Figs. 7 and 12). In HLM incubations, pathway 2 represented 75–80% of the 4'-N-methyl CN adduct formation (Table 1). In RLM incubations with KCN, it represented 60–70%. The percent of contribution by pathway 2 appeared to correlate with the extent of 4'-N-dealkylation of the
piperazines. The peak areas of 4'-N-dealkylation products from compounds X–XIV were much smaller than those from compounds III, IV, and VII, consistent with significantly lower levels of 4'-N-methyl piperazine CN adduct formation from compounds X–XIV (Tables 2 and 3). After normalizing the peak areas of 4'-N-dealkylations products and 4'-N-methyl piperazine CN adducts against parent peak areas in Tables 2 and 3 for HLMs and RLMs, respectively, the relationship between 4'-N-dealkylations and 4'-N-methyl piperazine CN adduct formation was plotted in Fig. 14, which clearly indicated that the extent of 4'-N-dealkylations was proportional to the level of 4'-N-methyl piperazine CN adduct formations and that the trends were similar in HLM and RLM incubations. When these compounds were incubated in liver microsomes with KCN, but in the absence of NADPH, no 4'-N-methyl piperazine CN adducts were detected.

**Incubation with [13C]-Labeled Potential Methyl Donors**

Two representative 4'-N-desmethyl piperazines I and IX were selected for the methyl donor experiment and to compare the extent of 4'-N-dealkylation products from human liver microsome incubations without potassium cyanide.

**TABLE 1**

Relative levels of 12C and 13C in 4'-N-methyl piperazine cyanide (CN) adducts from [13C]-labeled compounds (V) and (VIII) in microsomal incubations with the presence of potassium cyanide

<table>
<thead>
<tr>
<th>Parent Compound</th>
<th>Microsome</th>
<th>13C/12C Ratio of CN Adducts (A)</th>
<th>Pathway 1 Contribution (α)</th>
<th>Pathway 2 (Artifact) Contribution (1-α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II (standard)</td>
<td>None</td>
<td>0.28</td>
<td>0.24</td>
<td>0.76</td>
</tr>
<tr>
<td>V</td>
<td>HLM</td>
<td>0.62</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>V</td>
<td>RLM</td>
<td>0.94</td>
<td>0.17</td>
<td>0.83</td>
</tr>
<tr>
<td>VIII</td>
<td>HLM</td>
<td>0.49</td>
<td>0.30</td>
<td>0.70</td>
</tr>
<tr>
<td>VIII</td>
<td>RLM</td>
<td>0.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HLM, human liver microsome; RLM, rat liver microsome.

**TABLE 2**

Relationship of 4'-N-methyl piperazine cyanide (CN) adduct formation in human liver microsomes to the extent of 4'-N-dealkylation in piperazine compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent Piperazine</th>
<th>4'-N-Dealkylated Products*</th>
<th>4'-N-Methyl Piperazine CN Adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>Peak Area*a</td>
<td>m/z</td>
<td>Peak Area*b</td>
</tr>
<tr>
<td>III</td>
<td>449 690</td>
<td>421 25</td>
<td>460 58</td>
</tr>
<tr>
<td>IV</td>
<td>435 648</td>
<td>421 109</td>
<td>460 250</td>
</tr>
<tr>
<td>VII</td>
<td>455 471</td>
<td>441 18</td>
<td>480 52</td>
</tr>
<tr>
<td>X</td>
<td>467 62</td>
<td>453 0.6</td>
<td>492 0.1</td>
</tr>
<tr>
<td>XI</td>
<td>481 63</td>
<td>453 0.8</td>
<td>492 0.1</td>
</tr>
<tr>
<td>XII</td>
<td>495 107</td>
<td>453 0.7</td>
<td>492 0.1</td>
</tr>
<tr>
<td>XIII</td>
<td>495 60</td>
<td>453 0.3</td>
<td>492 1.0</td>
</tr>
<tr>
<td>XIV</td>
<td>509 79</td>
<td>453 0.4</td>
<td>492 0.1</td>
</tr>
</tbody>
</table>

*The dealkylation products were from human liver microsome incubations without potassium cyanide.

**TABLE 3**

Relationship of 4'-N-methyl piperazine cyanide adduct (CN) formation in rat liver microsomes to the extent of 4'-N-dealkylation in piperazine compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent Piperazine</th>
<th>4'-N-Dealkylated Products*</th>
<th>4'-N-Methyl Piperazine CN Adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>Peak Area*a</td>
<td>m/z</td>
<td>Peak Area*b</td>
</tr>
<tr>
<td>III</td>
<td>449 674</td>
<td>421 96</td>
<td>460 36</td>
</tr>
<tr>
<td>IV</td>
<td>435 722</td>
<td>421 247</td>
<td>460 273</td>
</tr>
<tr>
<td>VII</td>
<td>455 522</td>
<td>441 58</td>
<td>480 54</td>
</tr>
<tr>
<td>X</td>
<td>467 81</td>
<td>453 0.8</td>
<td>492 0.3</td>
</tr>
<tr>
<td>XI</td>
<td>481 99</td>
<td>453 1.4</td>
<td>492 0.3</td>
</tr>
<tr>
<td>XII</td>
<td>495 124</td>
<td>453 0.8</td>
<td>492 0.1</td>
</tr>
<tr>
<td>XIII</td>
<td>495 61</td>
<td>453 0.8</td>
<td>492 0.5</td>
</tr>
<tr>
<td>XIV</td>
<td>509 89</td>
<td>453 1.6</td>
<td>492 0.5</td>
</tr>
</tbody>
</table>

*The dealkylation products were from rat liver microsome incubations without potassium cyanide.

**Peak area was expressed as ion count per second (cps) × 10^6.**
methyl piperazine CN adduct formation. Compound I was from the tetrahydronaphthalene series, with its 4'-N-methyl piperazine (IV) showing extensive 4'-N-dealkylations in liver microsomal incubations. Compound IX was from the chroman series, with its 4'-N-methyl piperazine (X) showing less extensive 4'-N-demethylation. After the incubation of compound I or IX in HLM with KCN and [13C]-labeled potential one-carbon donors, the relative amount of [13C]methyl group incorporated into the CN adducts was evaluated by measuring the [13C]-labeled 4'-N-methyl piperazines CN adduct in the full-scan accurate mass spectrum. The theoretical protonated molecular ion of 4'-N-methyl piperazine CN adduct formed from I and IX should be at m/z 460 and 492 (Figs. 15A and 16A), respectively. However, in HLM incubations with [13C]-labeled potassium carbonate, methanol, or formaldehyde as the one-carbon donor, most of the 4'-N-methyl piperazine CN adduct ions formed were 1 amu higher at m/z 461 and 493 (Figs. 15, B–D and 16, B–D, respectively), suggesting the incorporation of the 13C isotope into the 4'-N-methylated CN adducts. The extent of 4'-N-methyl piperazine CN adduct formation was similar in both secondary amines (I and IX) but varied with the one-carbon donors (Fig. 17). Formaldehyde and methanol appeared to be more effective as one-carbon donors than potassium carbonate. After 30 minutes of HLM incubation in the presence of KCN and potassium carbonate as the one-carbon donor, only minor 4'-N-methyl piperazine CN adduct peaks were formed. However, when formaldehyde or methanol was used, significant levels of the parent compounds (I or IX) were converted to 4'-N-methyl piperazine CN adduct. Under current CN-trapping conditions with excess of one-carbon donor in the microsomal incubation, formaldehyde and methanol generated similar amounts of 4'-N-methyl piperazine CN adduct as measured by peak area counts (Fig. 17).

When I or IX was incubated in HLM with [13C]-labeled methanol or potassium carbonate in the presence of KCN, but in the absence of NADPH, no [13C]-labeled 4'-N-methyl piperazine CN adduct was detected; however, in the same HLM incubation, in the absence of NADPH, when [13C]-labeled formaldehyde was used, significant [13C]-labeled 4'-N-methyl piperazine CN adduct was formed (Fig. 18). When incubated with the same [13C]-labeled one-carbon donors in the presence of NADPH and KCN, but in the absence of HLM, similar results were observed (Fig. 19). When compound I was incubated in HLMs in the presence of [13C]-labeled potassium carbonate, KCN and the aldehyde trapping agent semicarbazide, CN adduction formation was significantly reduced (Fig. 20).

Discussion

In this study, several approaches were applied to investigate the mechanisms involved in the formation of 4'-N-methyl piperazine CN adducts. The first approach was the use of 4'-N-[13C]methyl piperazine to determine the extent of pathway 2 involvement. Results from this study showed that a significant proportion of the 4'-N-[13C]methyl groups in the piperazines was replaced by the natural [12C]methyl group in the CN adducts when incubated with liver microsomes in the presence of KCN. The second approach involved analysis of the relationship between the extent of 4'-N-dealkylation and the formation of 4'-N-methyl piperazine CN adduct. This study demonstrated that in 4'-N-alkyl piperazines, the level of 4'-N-methyl piperazine CN adduct formation was proportional to the extent of 4'-N-dealkylation. In a separate study, different one-carbon donors like [13C]-labeled methanol, formaldehyde, or potassium carbonate were evaluated to determine the carbon source for the 4'-N-methyl piperazine CN adduct formation. The theoretical natural 13C abundance in the piperazine CN adduct
standard (II) is 28%, which matches the observed $^{13}$C/$^{12}$C peak area ratio obtained in the mass spectrum (Fig. 4; Table 1). In 4'-N-[13C]methyl piperazines (V and VIII), if the 4'-N-methyl piperazine CN adduct was formed by direct reaction of the CN group with iminium ion after α-carbon oxidation (pathway 1), 100% of $^{13}$C isotope would be retained in the 4'-N-methyl group. In case the CN adduct was formed after 4'-N-dealkylation (pathway 2), the resulting 4'-N-methyl piperazine CN adduct would not contain the synthetic $^{13}$C carbon but rather the $^{12}$C carbon plus natural abundance (~28%) of $^{13}$C isotope in the molecule.

Our results showed that the 4'-N-methyl piperazine CN adducts (peak 3 in Figs. 7 and 12) from 4'-N-[13C]methyl piperazines V and VIII contained high levels of $^{12}$C carbon, indicating a significant contribution of pathway 2 to the 4'-N-methyl piperazine CN adduct formation.

The relationship of piperazine 4'-N-dealkylation with 4'-N-methyl piperazine CN adduct formation in the microsomal incubations was apparent when different 4'-N-substituted piperazines were compared. In microsomal incubations of the piperazine compounds containing a secondary 4'-nitrogen (I, VI, and IX) in the presence of KCN, the

![Fig. 16. Formation of 4'-N-[13C]methyl piperazine CN adduct in the HLM incubation of compound IX in the presence of KCN with [13C]-labeled one-carbon donors. (A) Simulated isotope spectrum for molecular ion of 4'-N-methyl piperazine CN adduct; (B–D) Isotope spectra for molecular ion of 4'-N-methyl piperazine CN adduct in HLM incubations with KCN in the presence of 5 mM of [13C]-labeled potassium carbonate, 1% (v/v) of [13C]methanol or 1% (v/v) of formaldehyde, respectively.](image)

![Fig. 17. Selected ion chromatogram on the extent of 4'-N-[13C]methyl piperazine CN adduct formation from compound I (left column) and IX (right column), respectively, in HLM incubations with KCN, including 5 mM [13C]-labeled potassium carbonate (top row), 1% (v/v) of [13C]methanol (middle row), or 1% (v/v) of [13C]formaldehyde (bottom row), respectively. Peak I, compound I at m/z 421.2607; peak I-MCN, 4'-N-[13C]methyl piperazine CN adduct of I at m/z 461.2733; peak IX, compound IX at m/z 453.2491; peak IX-MCN, 4'-N-[13C]methyl piperazine CN adduct of IX at m/z 493.2629; AA, integrated peak area (count per second).](image)
4'-N-methyl piperazine CN adducts were the predominant CN adducts. For 4'-N-alkyl piperazines (III, IV, VII, X–XII and XIV), the level of 4'-N-dealkylation was the rate-limiting step in pathway 2. The trend of 4'-N-dealkylation and 4'-N-methyl piperazine CN adduct formation was based only on ion chromatogram peak area without synthetic standard references, and the results presented are not fully quantitative.

To explore the potential carbon source for the formation of 4'-N-methyl piperazine CN-adducts, two 4'-N-desmethyl piperazine analogs (I or IX) were incubated in HLM with [13C]-labeled potential one-carbon donors such as potassium carbonate, methanol, or formaldehyde. All three [13C]-carbon sources resulted in the formation of 4'-N-[13C]methyl piperazine CN adducts from both I and IX in HLM incubations, thereby confirming that solvents such as methanol and formaldehyde or CO2 dissolved in the incubation mixture could be the one-carbon source that reacted with 4'-N-dealkylated piperazines and formed corresponding 4'-N-methylenepiperazine iminium ions.

In the Mannich reaction, the secondary amine reacts with aldehyde to generate reactive iminium ions that can be trapped by electron-rich alkenes (Tanaka et al., 2008; Abonia et al., 2012). Results from our study (Figs. 18 and 19) demonstrate that formaldehyde reacting with secondary alicyclic amines such as I and IX can generate 4'-N-methyl piperazine CN adducts in the absence of HLM or NADPH, indicating this to be a direct Mannich reaction. Methanol can be an efficient one-carbon source as it has been shown to be readily oxidized to formaldehyde in microsomal incubations (Teschke et al., 1974). We have shown in this study that formaldehyde in microsomal incubations (Teschke et al., 1974). We have shown in this study that formation of 4'-N-methyl piperazine CN adducts from the secondary alicyclic amines (I, IX) in the HLM incubation with methanol and KCN required NADPH, demonstrating that methanol oxidation to formaldehyde was likely the first step of the one-carbon addition to those secondary alicyclic amines. Formaldehyde would appear to be a more efficient one-carbon donor. However, since the current CN-trapping study was qualitative with an excess amount of one-carbon donors in the microsomal incubations, formaldehyde and methanol generated similar amounts of 4'-N-methyl piperazine CN adducts (Fig. 17), and the kinetics of these reactions remain to be determined.

Compared with formaldehyde and methanol, CO2 was less efficient as a one-carbon donor for the 4'-N-methyl piperazine CN adduct formation (Fig. 17). Primary and secondary amines have been reported to react with CO2 to form carbamic acid in different in vitro and in vivo systems (Schaefer, 2006). However, the mechanism of CO2 incorporation into alicyclic secondary amine to form the reactive iminium ion remains to be further investigated. Our preliminary 13C nuclear magnetic resonance analysis indicated that formation of carbamic acid was not detectable when compound I was directly reacted with [13C]-labeled potassium carbonate in aqueous solution (unpublished data). Furthermore, our results demonstrated that this one-carbon addition to the alicyclic secondary amines (I and IX) was NADPH dependent, suggesting the involvement of cytochrome P450 enzyme catalyzed bioactivation. We hypothesized that when using potassium carbonate as the one-carbon source in the liver microsomal incubation, the carbamic acid formation would be the first step of this methylation.

Fig. 18. Selected ion chromatogram on the extent of 4'-N-[13C]methyl piperazine CN adduct formation from compound I (left column) and IX (right column), respectively, in incubations without HLM, in the presence of NADPH and KCN, including 5 mM of [13C]-labeled potassium carbonate (top row), 1% (v/v) of [13C]methanol (middle row), or 1% (v/v) of [13C]formaldehyde (bottom row), respectively. Peak I, compound I at m/z 421.2607; peak IMCN, 4'-N-[13C]methyl piperazine CN adduct of I at m/z 461.2733; peak IX, compound IX at m/z 453.2491; peak IX-MCN, 4'-N-[13C]methyl piperazine CN adduct of IX at m/z 493.2629. Peak X, interference peak.
and this carbamic acid would need to be reduced to aldehyde then to alcohol, which would then form methylene iminium ion after dehydration. If the aldehyde intermediate was eliminated by a trapping agent like semicarbazide, it would likely reduce the iminium ion formation and thereby the $N$-methyl CN adduct. Our preliminary results indicate that semicarbazide could decrease the formation of 4'-$N$-methyl piperazine CN adducts in the HLM incubation of I with KCN and $[13C]$-labeled potassium carbonate, further supporting the hypothesis of aldehyde formation by piperazine 4'-$N$-carbamic acid reduction. Iminium ions from piperazine $\alpha$-carbon oxidation (pathway 1) have been considered a bioactivation process and those from pathway 2 as experimental artifact resulting from the potential interaction with formaldehyde in the incubation buffer (Gorrod and Sai, 1997; Li et al., 2006; Rousu and Tolonen, 2011; Barbara et al., 2012). However, the biologic and toxicological implications of iminium ions via pathway 2 require further investigation for the following reasons. First, in the case of methanol and carbon dioxide, cytochrome P450 enzymes are likely involved in the one-carbon addition to the alicyclic secondary amines, resulting in piperazine $N$-methylene iminium ion formation. Second, the potential one-carbon donors can be found in vivo since carbon dioxide is abundant as carbonate anion in the body. Methanol occurs naturally in the human body as a product of metabolism and through intake of fruits, vegetables, and alcoholic beverages (Shelby et al., 2004; Turner et al., 2006). Methanol levels in human blood range from 0.25 to 4.7 mg/l or 8 to 145 $\mu$M (Cook et al., 1991; Batterman and Franzblau, 1997). The endogenous concentration of formaldehyde in the blood of rat, monkey, and human is about 100 $\mu$M, and the level in rat liver is 2- to 4-fold higher (Heck and Casanova, 2004). The formaldehyde and methanol concentrations used in our study were 60 and 300 $\mu$M, respectively. Furthermore, the one-carbon addition products have been found in rat blood and urine dosed with homopiperazine (Martin et al., 2012). These naturally abundant one-carbon sources could potentially fuel the bioactivation of secondary amines to form $N$-methylene iminium ions in vivo. The resulting reactive $N$-methylene iminium ions could potentially bind to macromolecules, which is a concern because of potential liability for mechanism-based inactivation or idiosyncratic toxicity. Therefore, pathway 2 is relevant to in vivo bioactivation and warrants further investigations of its association to adverse drug reactions.

Fig. 19. Selected ion chromatogram on the extent of 4'-$N$-[13C]methyl piperazine CN adduct formation from compound I (left column) and IX (right column), respectively, in HLM incubations without NADPH, in the presence of KCN and including 5 mM $[13C]$-labeled potassium carbonate (top row), 1% (v/v) of $[13C]$methanol (middle row), or 1% (v/v) of $[13C]$formaldehyde (bottom row), respectively. Peak I, compound I at $m/z$ 421.2607; peak I-MCN, 4'-$N$-[13C]methyl piperazine CN adduct of I at $m/z$ 461.2733; peak IX, compound IX at $m/z$ 453.2491; peak IX-MCN, 4'-$N$-[13C]methyl piperazine CN adduct of IX at $m/z$ 493.262.

Fig. 20. Inhibitory effect of semicarbazide on 4'-$N$-[13C]methyl piperazine CN adduct formation in the HLM incubation of compound I with KCN in the presence of 5 mM $[13C]$-labeled potassium carbonate.
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Authorship Contributions

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References


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