Reaction of Homopiperazine with Endogenous Formaldehyde: A Carbon Hydrogen Addition Metabolite/Product Identified in Rat Urine and Blood

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ABSTRACT:

Drug reactivity and bioactivation are of major concern to the development of potential drug candidates in the pharmaceutical industry (Chem Res Toxicol 17:3–16, 2004; Chem Res Toxicol 19:889–893, 2006). Identifying potentially problematic compounds as soon as possible in the discovery process is of great importance, so often early in vitro screening is used to speed up attrition. Identification of reactive moieties is relatively straightforward with appropriate in vitro trapping experiments; however, on occasion unexpected reactive intermediates can be found later during more detailed in vivo studies. Here, we present one such example involving a series of compounds from an early drug discovery campaign. These compounds were found to react with endogenous formaldehyde from a rat in vivo study, resulting in the formation of novel +13-Da bridged homopiperazine products (equivalent to the addition of one carbon and one hydrogen atom), which were detected in urine and blood. The identification of these +13-Da products and their origin and mechanism of formation are described in detail through analyses of a representative homopiperazine compound [(N-(3-(3-fluorophenyl)-1,2,4-thiadiazol-5-yl)-4-(4-isopropyl-1,4-diazepane-2-carbonyl)piperazine-1-carboxamide [AZX]] by liquid chromatography-UV-mass spectrometry, 1H NMR, and chemical tests.

Introduction

Understanding the metabolic fate of putative drug candidates both in vitro and in vivo is a key component of drug discovery. Rapid production of early information describing the rate of clearance and site of metabolism is essential for directing iterative synthetic chemistry make-test cycles toward promising structural templates with the requisite properties for an effective drug. Reactive drug metabolites are of great concern in the pharmaceutical industry (Evans et al., 2004; Baillie 2006, 2009). Although their identification is relatively straightforward with appropriate in vitro trapping experiments, sometimes additional reactive compounds are found unexpectedly. In general, early metabolism studies involve incubation in hepatocytes or microsomes to mimic the most prevalent metabolic processes occurring in the liver. Incubate samples at t = 0 min and at a terminal time point (usually 30–60 min) are then compared by LC-UV-MS/MS. These studies can be both challenging and time-consuming even when only a small number of metabolites are identified. Simply mining the raw data to find the metabolites in the terminal sample often requires the use of a variety of techniques, ranging from simple UV comparison to complex common fragment searching (commonly referred to as broad band or MS²) or the use of sophisticated MS subtraction routines such as mass defect filtering. Experiments are therefore normally performed using state-of-the-art instruments offering a variety of options to aid detection and structure identification. The information is then used to direct and modify the chemistry toward compounds with favorable metabolic properties.

Improved understanding of bioactivation mechanisms, reactive intermediate formation (so-called reactive metabolites), and adverse toxicity has led to the front loading of biotransformation studies. In response, most pharmaceutical companies now employ reactive metabolite trapping screens using liver microsomes (usually human) fortified with nucleophiles such as GSH, cysteine, KCN, and methoxylamine (Prakash et al., 2008). The nucleophiles trap reactive electrophilic species at sufficient concentration to favor the formation of a stable product identified by their unique MS/MS spectral characteristics. Both early site of metabolism and reactive metabolite trapping studies rely on in vitro systems to generate the metabolites. However, metabolites can also be formed in complete biological systems, which could be missed if the metabolic pathways are unknown and/or the endogenous reagents are not represented in these in vitro systems. Hence, in our laboratories potential drug candidates undergo nonradiolabeled in vivo metabolite identification studies. These generally involve either a bile duct-cannulated study in rats, with collection of urine and bile over a 24-h period or collection of blood and urine from a high-dose rat pharmacokinetic study. The studies aid the identification of the excreted metabolites and ensure
identification of any unexpected reactive metabolites not generated or detected in the preliminary in vitro systems.

During recent rat in vivo metabolite identification studies with a series of lead compounds, cyclized GSH adducts were detected, similar to those reported by Doss et al. (2005), highlighting a potential reactive metabolite alert. This alert was not raised in the conventional GSH trapping screen, because of the type of the GSH rearrangement (data not shown). The reactophore in these lead compounds consisted of a terminal piperazine, which was responsible for this bioactivation. To preserve the potency of the compounds and remove the reactive metabolite risk, the chemistry was changed to a homopiperazine series.

Hence, several promising homopiperazine compounds from this series were dosed to rats to assess whether these GSH adducts were also formed. Instead, the analyses led to the observation of unusual, novel products with a molecular weight gain of 13 Da (while showing an apparent increase of +12 Da by mass spectrometry) as the major parent-related material in the urine and blood samples, which were not detected in the preliminary in vitro studies. The formation of these products, referred to as N-(3-(3-fluorophenyl)-1,2,4-thiadiazol-5-yl)-4-(4-isopropyl-1,4-diazepane-2-carbonyl)piperazine-1-carboxamide (AZX) + 13 throughout, is subject to further investigation in this article, with compound AZX (Fig. 1) as a representative structure for the “homopiperazine series.”

Materials and Methods

Chemicals and Suppliers. Compound AZX was synthesized and developed at AstraZeneca UK Ltd. (Macclesfield, UK). Acetonitrile (ACN), methanol (MeOH), ammonium acetate (analytical reagent grade), and formic acid were acquired from Thermo Fisher Scientific (Loughborough, UK). Potassium cyanide, formaldehyde (37% in H₂O), and the deuterated NMR solvents were sourced from Sigma-Aldrich (Poole, UK).

Standard/Stock Solutions. AZX stock solution. AZX was dissolved in MeOH at a concentration of 200 μM.

AZX test solution. AZX was dissolved in MeOH/water (30:70%, v/v) to a concentration of 10 μM. The test solution was prepared from the stock solution. (Equivalent stock and test solutions were prepared in ACN to assess whether MeOH was a contributing factor in AZX formation.)

Formaldehyde. Formaldehyde (37% in H₂O) was used undiluted in all spiking experiments.

KCN. KCN was prepared in water to a concentration of 50 mM.

Animal Dosing and Sample Collection (Urine and Blood). Dosing solution. AZX was prepared in a formulation of dimethylamine-water (40:60, v/v) at a concentration of 1 mg/ml.

Rats. Male Han Wistar rats (n = 4) were divided into two groups (n = 2/group), each receiving a single dose of AZX at 2 mg/kg i.v. at a dose volume of 2 ml/kg. Urine was collected predose and at 0 to 6, 6 to 12, and 12 to 24 h from group 1, whereas group 2 provided blood via the tail vein predose and at 20 min, 1.5 h, 6 h (at a volume of 0.3 ml), and finally 24 h (at a volume of 1.3 ml). Rat urine and blood were stored frozen at −20°C until further analysis. Additional predose/control rat urine samples were provided on request, throughout the study.

Sample Preparation. Urine. Urine samples (0–24 h postdose) were pooled using a set volume (100-μl aliquots) from each time point before analysis. Predose urine (190- and 90-μl aliquots) was spiked with 10 μl of the AZX stock solution (200 μM) to give a final concentration of 10 and 20 μM, respectively. Both the predose and 0 to 24 h urine samples were centrifuged at approximately 12,000g for 5 min before analysis. The supernatant was then
transferred to Agilent high-performance liquid chromatography 2-ml vials with 200-μl inserts for LC-MS analysis.

**Blood.** Blood samples (0–24 h postdose) were pooled using a set volume (50-μl aliquots) from each time point. Blank/predose rat blood was spiked with 10 μl of the AZX stock solution (200 μM). Both the predose/blank and 0 to 24 h blood were diluted 1:1 with H₂O and quenched with chilled (4°C) ACN (1:3, v/v) followed by centrifugation at 12,000g for 5 min, and the supernatant was transferred to Agilent high-performance liquid chromatography 2-ml vials for LC-MS analysis.

**Formaldehyde Addition.** The formaldehyde-spiked AZX test solution was prepared by adding 10 μl of formaldehyde (37% in water) to 500 μl of the AZX test solution (10 μM, MeOH/H₂O, 30:70, v/v) and analyzed by LC-MS immediately.

**Potassium Trapping Experiments.** Ten-microliter aliquots of the KCN solution (50 mM KCN/water) were added to a) 90 μl of the 0 to 24 h rat urine pool, b) 90 μl of predose rat urine, which was spiked with 10 μl of AZX (200 μM, i.e., the stock solution) to give a final concentration of 20 μM, and c) 90 μl of the formaldehyde-spiked AZX test solution (detailed above). Both the urine samples and the formaldehyde-spiked AZX test solution were left at room temperature for 24 h before injection onto the LC-MS system.

**Preparation of NMR Samples.** Here, a more concentrated solution of AZX was prepared, at a concentration of 1 mM in ACN. For NMR spectroscopy, three solutions were prepared: a) 2 ml of AZX/ACN (1 mM); b) solution a with 100 μl of formaldehyde (37% in H₂O); and c) 1 ml of solution b with 100 μl of KCN (50 mM). Each of the solutions was stored for 24 h (to allow complete formation of the products) before evaporation of the solvents. The solvents were evaporated under nitrogen; each sample was freeze-dried and reconstituted in 200 μl of MeOH-d₄ for 1H NMR spectroscopic analysis.

**Identification and Structural Characterization of Azoxystrobin + 13 by UPLC-LTQ Orbitrap.** Accurate mass structural characterization was acquired on a LTQ Orbitrap XL connected to a Waters Acuity UPLC system. The Waters Acquity system consisted of a binary UPLC PUMP, a column oven, an autoinjector, and a photodiode array detector. Separations were performed on a Kinetix C18 column (100 × 2.0 mm, 2.6 μm; Phenomenex, Macclesfield, UK) preceded by a guard filter in an column oven at 50°C. The mobile phase consisted of different solvent systems to assess the contribution of solvent/metabolite interactions. For the in vivo samples, the AZX + 13 product was detected at approximately 95:5 (m/z 488: AZX parent) (data not shown).

**Detection of the Novel Metabolite with m/z 488 (AZX + 13) in the In Vivo Samples.** Typically, in early in vivo metabolite identification studies, temporal blood and urine samples are typically combined to obtain a single pooled sample for each matrix. The sample is then analyzed on a LC-UV-MS/MS system in conjunction with a predose sample spiked with analyte to obtain a final concentration of 10 μM (a concentration shown to produce a discernible UV response in the biological matrix).

**SPiking of the predose serves two purposes:** it helps to rule out synthetic impurities that could be misinterpreted as metabolites and to discount endogenous material visually or by automated data subtraction.

In this study, only low levels of compound AZX were detected upon analysis by UPLC-UV-MS/MS in both the 0 to 24 h rat urine-pooled sample and the AZX-spiked predose urine sample. However, an unexpected major parent-related peak was detected with a molecular ion of m/z 488. This observation was confirmed by spiking of parent compound into samples of fresh predose/control urine (representing final AZX concentrations of 10 and 20 μM), which, once again, resulted in the spontaneous formation of the m/z 488 product as the major parent-related component, at an average ratio of approximately 95:5 (m/z: 488: AZX parent) (data not shown).

It was initially assumed that either the wrong compound was dosed or that the parent had either degraded/formed chemical adducts, because the molecular ion, i.e., the mass addition, could not be explained. Therefore, to verify the identity and stability of the parent, the solvent standards (i.e., the 200 μM AZX stock solution and the 10 μM AZX test solution) and the actual dose solution (diluted to 10 μM in 30:70, methanol/water, v/v) were analyzed in conjunction with repeat predose and 0 to 24 h rat urine samples. A comparison of the UV chromatograms (extracted at λ = 240–245 nm) for the AZX-
spiked predose urine samples (at 10 and 20 μM final concentrations) and the AZX solvent standards showed that in urine the UV peak for AZX (RT = approximately 5.32 min) was depleted, whereas the m/z 488 product (RT = approximately 4.45 min) was abundant (Fig. 2). However, the AZX solvent standards and the dose solution resulted in the observation of the correct molecular ion of [M + H]⁺ 476, confirming that the compound had not degraded in solution over time.

Similar observations were made with the 0 to 24 h rat blood, in which the m/z 488 product was also observed, although at a reduced amount (at an approximate ratio of 40:60, m/z 488: AZX) as assessed by UV (data not shown). Furthermore, the spiking experiments with predose blood, as conducted with the urine samples, again confirmed the formation of the m/z 488 product. From these data it was surmised that AZX was reacting with a component present in the urine and blood, which is the subject of this investigation.

A thorough review of the 0 to 24 h sample data (urine and blood) confirmed that this m/z 488 metabolite/product (RT = approximately 4.45 min), represented the majority of the AZX-related material in urine as determined by UV and mass spectrometry. The mass of the m/z 488 product was equivalent to an increase of 12 Da from parent (AZX, [M + H]⁺ 476), although it represented an actual increase of 13 Da when molecular weights were compared (as detailed in the following section).

**Structural Characterization of the Novel Metabolite/Product, m/z 488 (AZX + 13) by UPLC-MS/MS.** From accurate mass measurement this apparent metabolite was determined to have a mono-isotopic mass of 488.2242, almost exactly an increase of 12.0000 Da over the parent [M + H]⁺ 476.2243, suggesting addition of one carbon atom. This was further substantiated after an elemental composition analysis in which no rational alternative molecular formula could be identified.

The proposed fragmentation pattern and LTQ Orbitrap HCD MS/MS spectrum of this metabolite/product (RT = approximately 4.45 min) (Fig. 3) contained diagnostic fragment ions m/z 267.2184 and m/z 153.1386. These ions corresponded to the addition of 12 Da to the key fragments m/z 255 and m/z 141 in the MS/MS spectrum of AZX. The observation of the fragment ions m/z 267.2184 and m/z 153.1386 with accurate mass (±2 ppm) and elemental composition analysis appeared to confirm the addition of a single carbon atom to the homopiperazine ring. The exact position of the carbon addition could not be determined by MS; however, it was possible to postulate the structure as a bridged homopiperazine (as shown in Fig. 3).

**Difference between Theoretical Molecular Weight and Measured Molecular Ion of the Proposed Bridged Homopiperazine.** The proposed bridged structure equates to the addition of one carbon and one hydrogen atom, i.e., a gain of 13 Da, which is inconsistent with the 12-Da increase as determined by the mass spectrometry data. However, because the bridged product has a fixed permanent positive charge (M⁺), it cannot produce a protonated molecular ion [M + H]⁺ by positive ion electrospray (+ESI). Hence, whereas the calculated nominal mass of the parent (AZX) is 475 and the mass measured by +ESI mass spectrometry ([M + H]⁺) is 476 (Fig. 1), the calculated nominal mass and the mass measured by +ESI MS of the bridged ion are both 488. Therefore, a comparison of the theoretical molecular weights of AZX and AZX + 13 results in a mass difference of 13 Da, compared with the 12-Da difference in the measured molecular ions by mass spectrometry. To highlight the +13 Da addition of this structurally unique product compared with the +12 carbon addition products reported in the literature (see Discussion), the product is referred to as AZX + 13.

**KCN Addition as a Chemical Test to Confirm the Presence of a Quaternary Nitrogen.** Cyanide chemically forms adducts with iminium ions and is used widely in reactive metabolism trapping.
studies in biological samples across the pharmaceutical industry (Argoti et al., 2005). To confirm the presence of a quaternary nitrogen (i.e., the bridged homopiperazine moiety as shown in Fig. 3), KCN was added to the AZX-spiked predose and the pooled 0 to 24 h urine sample. On addition of KCN, the AZX\textsubscript{13} product peak ($m/z$ 488, RT approximately 4.45 min) reduced in size (based on its UV response), whereas an additional peak [M + H]\textsuperscript{+} 515 (RT = approximately 5.85) was detected in each of the samples (Fig. 4). Further investigation by MS/MS fragmentation determined the addition of 27 Da (CN) on the homopiperazine ring, by the presence of the key fragment ions $m/z$ 294.2289 and $m/z$ 180.1497, corresponding to +27 Da on $m/z$ 267.2179 and $m/z$ 153.1386, respectively. Confirmation of the presence of the quaternary nitrogen on the homopiperazine led to the investigation of the mechanism of formation of AZX + 13.

**Investigation of the Formation of AZX + 13.** On the basis of the MS results and the KCN trapping experiment (confirming the quaternary nitrogen), it appeared that AZX was reacting with a component in the urine to produce a one carbon and one hydrogen atom addition bridged homopiperazine.
The same product was observed with pooled blood, albeit to a lesser extent. Formaldehyde, reported to occur naturally in living systems (Heck and Casanova, 2004), was suggested as a likely candidate to generate AZX + 13, via a quaternary Schiff base intermediate (iminium ion), which is then intramolecularly stabilized by forming the bridged homopiperazine (as shown in Fig. 5).

To test this hypothesis, formaldehyde (10 μl, 37% in H₂O) was spiked into the AZX test solution (10 μM, 500 μl), which was analyzed immediately on the UPLC-MS system. A product was formed at nearly 100% yield within the time taken to inject the sample, confirming that formaldehyde reacts rapidly with AZX at room temperature. This chemical product was verified as the AZX + 13 product, which was identical to that detected in the biological samples (urine and blood), as assessed by chromatographic retention time, UV, accurate mass, and MS/MS fragmentation. This now provided an efficient means to generate AZX + 13 at a sufficient scale for full structural confirmation by ¹H NMR spectroscopy, as detailed under Methods and Materials. Not only was the confirmation of the bridged homopiperazine structure of importance, but the resultant structure of the AZX + 13 CN adduct was also of interest to confirm the exact position of the CN addition, in view of several possible isomeric products.

Confirmation of AZX + 13 and the AZX + 13 + CN Adduct by ¹H NMR Spectroscopy. After identification by MS, the structures of the parent (AZX), the AZX + 13 product, and the AZX + 13 + CN adduct were verified by ¹H NMR spectroscopy (Fig. 6, A, B, and C, respectively). After the addition of formaldehyde, no changes in the spectra were observed in the aromatic regions of AZX and AZX + 13 spectra, indicating that the site of modification is remote (data not shown).

However, the aliphatic region highlighted several chemical shift changes supporting the proposed formation of the bridged homopiperazine (Fig. 6, A and B). These mainly comprised changes in the chemical shifts of the protons on the homopiperazine moiety, such as the isopropyl methyl doublets, which have shifted from 0.94 and 0.98 to 1.34 and 1.38 ppm, respectively. Likewise, all the residual homopiperazine protons experienced a shift to higher frequency. In addition to the chemical shift changes, two doublets (labeled Ha and Hb), not initially present in the parent spectrum, were observed at 4.68 and 3.91 ppm, with a coupling constant of J = 9.82 Hz and an integral value of one proton per doublet, which showed a clear connectivity in the two-dimensional COSY spectrum (data not shown). The suggested structure is consistent with the proposed one carbon bridge across the homopiperazine.

Figure 6C shows the ¹H NMR spectrum of the AZX + 13 + CN adduct. The −CN addition was shown to have largely reversed the chemical shift changes induced by the addition of formaldehyde (the formation of the bridged homopiperazine), yet an additional set of doublets (an AB system, labeled Hc and Hd) was noted at 3.7 and 3.5 ppm, which showed a strong connectivity in the two-dimensional COSY spectrum (data not shown). On the basis of this evidence, two isomeric possibilities could be suggested (Fig. 7, isomers 1 and 2). Selective irradiation experiments were used to determine the correct structure of the AZX + 13 + CN adduct.

The selective ROESY experiment, irradiating the isopropyl methyl signals (labeled 8.8‴), showed clear through-space interactions to the isopropyl proton (labeled 9 in the structure), as well as the homopiperazine protons 2 and 3 (Fig. 7). There was no enhancement of the bridged protons (labeled a and b), indicative of the six-membered ring (isomer 2), hence providing evidence that this was not the preferred structure. Instead, the spectral data supported the presence of the NCH₂CN side chain (isomer 1), after addition of KCN.

Discussion

This study demonstrated that compound AZX and various analogs containing a homopiperazine moiety reacted rapidly with formaldehyde in biological matrices to form a carbon and hydrogen addition bridged homopiperazine (+13-Da products). However, the origin/source of formaldehyde was still unclear, because it could have been endogenous, as reported by Heck and Casanova (2004) or, indeed, derived from the solvent system (methanol or formic acid), although it was shown to form irrespective of the mobile phase combination or spiking solvent used. A series of publications have described the formation of a +12-Da product from a reaction with formaldehyde, which was suggested to have derived from methanol, as discussed below.

It has to be pointed out that in these articles the reaction with formaldehyde resulted in +12-Da heterocyclic products (having gained one carbon atom over their respective parent compounds), whereas our example, through a different mechanism, yielded a novel bridged homopiperazine with a quaternary nitrogen (after addition of one carbon and one hydrogen). Hence, Yin et al. (2001) described the addition of +12 Da to the parent compound identified in/derived from in vitro experiments, in which S9 fractions/hepatic incubations were spiked with an analyte in methanol. However, it was concluded that the MeOH was first metabolized to formaldehyde (Teschke et al., 1974), which then reacted with a basic group on the incubated compound. Each of the compounds tested, containing either 1,2-amino hydroxyl or 1,2-diamino reacted with the metabolic formalde-
hyde to generate ring-closed heterocyclic or +12-Da products. Cunningham et al. (1990) reported a similar problem with diaminotoluene reacting with formaldehyde generated from the use of methanol as a spiking solvent for in vitro incubations. The product was formed through cross-linking of two molecules of 2,4-diaminotoluene with formaldehyde to give bis(2,4-diamino-5-tolyl)methane.

Köppel et al. (1991) described the formation of +12-Da products from the gas chromatographic analyses of several drugs by addition of formaldehyde and subsequent loss of water. It was suggested that the formaldehyde was formed by thermal degradation of MeOH in the gas chromatographic source.

These reported cases describe an analytical or in vitro artifact from the use of MeOH as a diluent or solvent, which is then believed to be oxidized chemically or metabolically to formaldehyde. In this study AZX + 13 (the formaldehyde addition product) was derived directly from an in vivo investigation, suggesting a potentially different (i.e., natural) source of formaldehyde for the reaction. AZX + 13 was not detected in any preliminary in vitro experiments because test compounds are typically spiked into incubations using ACN and not MeOH. However, we have also demonstrated that the MeOH used as mobile phase constituent and/or indeed as the AZX diluent (used in the stock and test solutions) did not cause the formation of AZX + 13.

Further investigations were performed to rule out MeOH as the source of the formaldehyde or indeed as the reagent itself. The experiments are not described in detail here; however, a brief summary is provided.

On spiking of compound AZX/MeOH into “freshly collected” predose/control rat urine, using the MeOH/H2O gradient, AZX + 13 was produced spontaneously with 90 to 100% yield (based on the UV response) and remained constant over 1 month (with the urine stored at 4°C). However, on spiking of AZX/MeOH into “old” predose/control rat urine, there was very little evidence of the formation of the AZX + 13 product. Here, the urine was stored cold (at 4°C) for approximately 2 months before spiking, allowing degradation/evaporation of the formaldehyde, on the basis of the half-life of formaldehyde (Organization for Economic Cooperation and Development Screening Information Data Set, Formaldehyde, 2002, http://www.inchem.org/documents/sids/sids/FORMALDEHYDE.pdf). After the 2-month aging period, upon spiking of AZX, only approximately 5 to 10% of the product was formed, the ratio remaining constant over a further month.

Additional evidence on the formation of the in vivo AZX + 13 was provided by spiking fresh rat urine samples with AZX, as conducted in the experiments detailed above, using ACN as the spiking solvent. The UPLC-UV-MS/MS analysis was also performed in the
absence of MeOH, using ACN (containing 0.1% formic acid) as the mobile phase, revealing that AZX + 13 was still formed as the major product (between 90 and 100%), as assessed by UV quantification and MS analysis (data not shown). However, the AZX standards (the stock and test solutions, prepared in ACN) did not produce the product when subjected to UPLC-UV-MS/MS analysis, ruling out ACN and formate as possible reagents.

These simple experiments suggested not only that the formaldehyde in the urine was naturally present but also that it was depleted over time. In addition, they proved that AZX + 13 was not likely to be an analytical artifact from the MeOH, ACN, or formate used in the analyses.

The findings from our research appear to support the evidence in the literature, namely that the formaldehyde occurs naturally in all mammalian tissues, cells, and bodily fluids and that it is present in rat blood at approximately, 0.1 mM concentration (Heck and Casanova, 2004). Formaldehyde and its oxidation product formate are reported to be key intermediates in the “one-carbon pool” (Neuberger, 1981). Certain xenobiotics such as MeOH, N-, O-, or S-Me compounds, and methylene chloride apparently can also contribute to this pool. The one-carbon pool is used for the biosynthesis of purines, thymidine, and certain amino acids, which are incorporated into the DNA, RNA, and proteins during macromolecular synthesis. It is therefore suggested that compound AZX scavenges endogenous formaldehyde in vivo, similar to aminoguanidine, which was reported by Kazachkov et al. (2007).

Formaldehyde is known to be responsible for cross-linking proteins (e.g., Metz et al., 2004; Toews et al., 2008) and some xenobiotics (Cunningham et al., 1990) through a Schiff base intermediate. Considering that basic amine groups are ubiquitous on many xenobiotics and proteins, it is worth noting the potential for formaldehyde to cause cross-linking between xenobiotics and proteins in vivo. Hence, susceptibility to formaldehyde addition via a direct or metabolic route could lead to the formation of covalently bound protein adducts. However, the reaction in vitro relies on the presence of MeOH, e.g., used as spiking solvent, as a source of the formaldehyde [as outlined by Yin et al. (2001) and Cunningham et al. (1990)]. Therefore, the potential for some basic xenobiotics to covalently bind to protein may not be flagged by reactive metabolite screening or radiolabeled in vitro covalent binding studies.

Here, the Schiff base reactive intermediate, trapped because of the proximity of the two nitrogens (as in a twisted boat conformation), formed a very stable bridged homopiperazine detectable by LC-UV-MS. However, in most other basic xenobiotics the Schiff base intermediate would not be intramolecularly trapped to form a stable product detectable by subsequent analyses. Hence, there is potential for this reactive intermediate to be missed in in vivo/in vitro metabolite identification studies, even when formaldehyde is present.

Because the initial terminal piperazine series was found to generate electrophilic, reactive intermediates metabolically, the homopiperazine series was thought to provide a safer suitable alternative. Therefore, the formation of a bridged homopiperazine through a reactive quaternary Schiff base intermediate was an unexpected observation. The homopiperazine ring is neither planar nor symmetrical, so the conformation adopted clearly proved favorable for the formation of a one-carbon bridge.

Various other homopiperazine analogs of compound AZX have also been investigated for +13-Da product formation and as demonstrated in this study with compound AZX, these products have been formed rapidly in vivo, as well as being confirmed in spiking experiments with formaldehyde (data not shown). These studies have highlighted the fact that the homopiperazine moiety appears to be a highly reactive group, and the observations made in this study support the decision to change the chemistry of these compounds.

In conclusion, this study has demonstrated that compound AZX and various analogs containing the homopiperazine moiety reacted rapidly with formaldehyde present in biological fluids, as a constituent of the one-carbon pool, suggesting that it is a metabolite, as well as a chemical product. The homopiperazine moiety was initially included in the lead compounds as an inert/unreactive alternative to the terminal piperazine, which was shown to be prone to bioactivation and subsequent covalent binding with GSH. Hence, the addition of the terminal homopiperazine group was believed to retain the potency of the compounds while decreasing their reactivity. However, as we have shown in this study, this group is itself highly reactive with endogenous formaldehyde, forming bridged homopiperazines.

The findings in this article suggest that compound AZX and its homopiperazine analogs scavenge endogenous formaldehyde in vivo similar to aminoguanidine, which was reported by Kazachkov et al.
What appears to be a desired effect with aminoguanidine proved to be a toxicity alert with the AZX-derived compounds. Apart from the reactivity aspect itself, quantitation of parent drug in blood and urine would be greatly compromised, creating problems for lead optimization and pharmacokinetic assessments. In addition, the formation of this adduct could artificially distort metabolite profiles in human metabolism studies, affecting (metabolites in safety testing) investigations (U.S. Food and Drug Administration guidance on Safety Testing of Drug Metabolites, 2008, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf).

However, overall the study demonstrated that the bridged homopiperazine was readily formed in vivo, producing a stable product/metabolite, which did not degrade in urine for at least 1 month. This finding has provided a suitable method and an easy and highly efficient means (>95% yield) for the generation of a bridged homopiperazine synthetically by a simple reaction with formaldehyde.

**Authorship Contributions**

*Participated in research design:* Martin, Lenz, Temesi, Wild, and Clench.

*Conducted experiments:* Martin and Lenz.

*Contributed new reagents or analytic tools:* Martin and Lenz.

*Performed data analysis:* Martin and Lenz.

*Wrote or contributed to the writing of the manuscript:* Martin, Lenz, Temesi, Wild, and Clench.

**References**


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