Reaction of homopiperazine with endogenous formaldehyde. A carbon hydrogen addition metabolite/product identified in rat urine and blood.

Scott Martin, Eva M. Lenz, Dave Temesi, Martin Wild and Malcolm R. Clench

DMPK Department, Alderley Park, AstraZeneca UK Ltd., Macclesfield, Cheshire SK10 4TG, United Kingdom (S.M., E.M.L., D.T., M.W.)

Biomedical Research Centre, Sheffield Hallam University, Howard Street, Sheffield S1 1WB, United Kingdom (M.R.C.)
Running title: Reaction of homopiperazine with formaldehyde.

Corresponding author:
Scott Martin
DMPK Department,
Alderley Park,
AstraZeneca UK Ltd.,
Macclesfield,
Cheshire SK10 4TG,
United Kingdom
Tel. +44-1625-518479
Fax: +44-1625-230614
e-mail: Scott.Martin2@astrazeneca.com

Number of Text Pages:
Number of Tables: 0
Number of Figures: 7
Number of References: 17
Number of words in the abstract: 181
Number of words in the introduction: 651
Number of words in the discussion: 1462

List of non-standard abbreviations:
LC, liquid chromatography
UPLC, Ultra high-Performance Liquid Chromatography
MS, Mass spectrometry
KCN, Potassium cyanide
HCD, Higher energy Collisional Dissociation
ESI, ElectroSpray Ionisation
+ESI, Positive ion ElectroSpray Ionisation
Abstract

Drug reactivity and bioactivation are of major concern to the development of potential drug candidates in the pharmaceutical industry (Evans et al., 2004, Baillie 2006). Identifying potentially problematic compounds as soon as possible in the discovery process is of great importance, so often early in vitro screening is employed 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 1 carbon and 1 hydrogen atom), which were detected in urine and blood. The identification of these +13 Da products, their origin and mechanism of formation are described in detail through analyses of a representative homopiperazine compound (AZX) by Liquid Chromatography (LC)-UV-Mass Spectrometry (MS), $^1$H Nuclear Magnetic Resonance 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 are essential for directing iterative synthetic chemistry make-test-cycles towards 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 and 2009). Although their identification is relatively straightforward with appropriate in vitro trapping experiments, sometimes additional reactive compounds are found unexpectedly. Generally, 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 mins and at a terminal time point (usually 30–60 mins) are then compared by LC-UV-MSMS. These studies can be both challenging and time consuming even when identifying only a small number of metabolites. 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 MS3) or the use of sophisticated MS subtraction routines such as mass defect filtering. Experiments are therefore normally carried out using state of the art instruments offering a variety of options to aid detection and structure identification. The information is then
utilized to direct and modify the chemistry towards compounds with favourable metabolic properties.

The improved understanding of bioactivation mechanisms, reactive intermediate formation (so called reactive metabolites) and adverse toxicity has lead 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 glutathione (GSH), cysteine, potassium cyanide (KCN) and methoxylamine (Prakash et al., 2008). The nucleophiles trap reactive electrophilic species at sufficient concentration to favour the formation of a stable, product identified by their unique MSMS 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 that 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 non-radiolabelled in vivo metabolite identification studies. This generally involves either a bile-duct cannulated study in rats, collecting urine and bile over a 24 h period, or collecting blood and urine from a high dose rat pharmacokinetic study. These 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, cyclised 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, due to 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. In order 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 in order to assess whether these GSH adducts were also formed. Instead, the analyses led to the observation of unusual, novel products with a MW gain of 13 Da (whilst 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 AZX+13 throughout, is subject to further investigation in this paper, with compound AZX (Figure 1) as a representative structure for the ‘homopiperazine series’.
Methods

Chemicals and suppliers

Compound AZX, N-(3-(3-fluorophenyl)-1,2,4-thiadiazol-5-yl)-4-(4-isopropyl-1,4-diazepane-2-carbonyl)piperazine-1-carboxamide, 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 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, in order to assess if MeOH was a contributing factor of AZX+13 formation)

Formaldehyde: Formaldehyde (37% in H₂O) was used neat 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 2 groups (n=2/group), each receiving a single dose of AZX at 2 mg/Kg intravenously (iv) at a dose volume of 2 mL/Kg. Urine was collected pre-dose and at 0-6 h, 6-12 h and 12-24 h from group 1, whilst group 2 provided blood via the tail vein pre-dose and at 20 min, 1.5 h, 6 h (at a volume of 0.3 mL) and finally at 24 h (at a volume of 1.3 mL).

Rat urine and blood were stored frozen at –20 ºC until further analysis. Additional pre-dose/control rat urine samples were provided on request, throughout the study.

Sample preparation

Urine: Urine samples (0-24 h post-dose) were pooled using a set volume (100 μl aliquots) from each time point prior to analysis. Typically, pre-dose urine (190 μL and 90 μL aliquots) was spiked with 10 μL of the AZX stock solution (200 μM) to give a final concentration of 10 μM and 20 μM, respectively. Both the pre-dose and 0-24 h urine samples were centrifuged at ca. 12000 g for 5 min prior to analysis. The supernatant was then transferred to Agilent HPLC 2 mL vials with 200 μL inserts for LC-MS analysis.

Blood: Blood samples (0-24 h post-dose) were pooled using a set volume (50 μL aliquots) from each time point. Blank/pre-dose rat blood was spiked with 10 μL of the AZX stock solution (200 μM). Both the pre-dose/blank and 0-24 h blood were diluted 1:1 with H2O and quenched with chilled (4ºC) ACN (1:3 v/v) followed by centrifugation at
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12000 g for 5 min and the supernatant transferred to Agilent HPLC 2 mL vials for LC-MS analysis.

**Formaldehyde addition**

Preparation of the formaldehyde-spiked AZX test solution: 10 μL of formaldehyde (37% in water) was added to 500 μL of the AZX test solution (10 μM, MeOH/H₂O, 30/70, v/v) and analysed by LC-MS immediately.

**Potassium Trapping Experiments**

10 μL aliquots of the KCN solution (50mM KCN/water) were added to:

a) 90 μL of the 0-24 h rat urine pool

b) 90 μL of pre-dose 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.

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 prior to injection onto the LC-MS system.

**Preparation of NMR samples**

For NMR spectroscopy, a more concentrated solution of AZX was prepared, at a concentration of 1 mM in ACN.
For NMR spectroscopy, 3 solutions were prepared:

a) 2 mL of AZX/ACN (1 mM)

b) Solution (a) with 100 μL of formaldehyde (37% in H₂O)

c) 1 mL of solution (b) with 100 μL of KCN (50 mM).

Each of the solutions were stored for 24 h (to allow complete formation of the products) prior to 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 AZX+13

UPLC-LTQ-Orbitrap

Accurate mass structural characterisation work was acquired on a LTQ Orbitrap XL connected to a Waters Acquity UPLC (Ultra high-Performance Liquid Chromatography). The Waters Acquity system consisted of a binary UPLC PUMP, Column oven, autoinjector equipped and a photodiode array detector. Separations were carried out on a Kinetix C18 (100 x 2.0 mm, 2.6 μM, Phenomenex, Macclesfield, UK) preceded by a guard filter in an column oven at 50 ºC. The mobile phase consisted of different solvent systems, in order to assess the contribution of solvent/buffer/acidifier to the AZX+13 formation:
a) ammonium acetate (5 mM, eluent A) and methanolic ammonium acetate (5 mM, eluent B).

b) Formic acid (0.1%, eluent A) and formic acid/ACN (0.1%, eluent B).

The AZX stock and test solution were found to be stable in both mobile phase systems. For the in vivo samples, the AZX+13 product was detected at approximately the same concentrations in either solvent system used. Hence, solvent system (a) was subsequently used routinely.

The elution profile was: linear gradient 90% A to 10% A, 0.00 mins to 8.00 mins.; isocratic hold, 10% A 8.00 mins to 10.00 mins; re-equilibration 90% A, 10.01 mins to 13.00 mins. The flow rate was 0.6 mL/min and the eluent was introduced into the mass spectrometer via the LTQ divert valve at 1 min. The injection volume was 20 μL and UV spectra were acquired over 190-330 nm. The LTQ-Orbitrap XL was equipped with an electrospray ionisation (ESI) source (Thermo Fisher Scientific, Bremen, Germany) which was operated in positive mode. Source settings were: Capillary temperature 350 ºC, sheath gas flow 25, auxiliary gas flow 17, sweep gas flow 5, source voltage 3.5 kV, source current 100.0 μA, capillary voltage 18 V, tube lens 75.0 V. Full scan MS data were obtained over the mass range of 100 to 1000 Da at a peak resolution of 7500. Targeted MSMS experiments were acquired using Higher energy Collisional Dissociation (HCD) fragmentation, isolation width 2 Da, normalised collision energy 45, and activation time 30 ms. HCD fragment ions were monitored by the Orbitrap using 7500 resolution. LTQ and Orbitrap mass detectors were calibrated within one day of commencing the work using Proteomass LTQ/FT-Hybrid ESI positive mode calibration mix (Supelco Bellefonte USA).
1H NMR Spectroscopy for Structure Verification

1H NMR analyses were carried out in order to confirm and structurally characterize AZX+13 and the AZX+13+CN adduct.

1H NMR analysis was performed on a Bruker AVANCE 600 MHz spectrometer, operating at 600.13 MHz 1H resonance frequency. The NMR spectrometer was equipped with a 2.5 mm SEI 1H/19F probe.

Typically, 1D 1H NMR spectra of substrate and product were acquired without solvent suppression into 65k data points over a spectral width of 12376 Hz, resulting in an acquisition time of 2.64 s. A relaxation delay of 2.4 s was employed to ensure T1 relaxation between successive scans and, depending on concentration, approximately 64-512 scans were acquired per sample.

2D 1H-1H COSY (COrelation SpectroscopY, gradient enhanced, Bruker Biospin Ltd.) experiments were employed on the samples to determine signal connectivities. Here, spectra were acquired into 4k data points in F2, and 128 increments in F1. The spectral width was set to 8012 Hz, resulting in an acquisition time of 0.26 s. A relaxation delay of 1.5 s was employed between successive scans, 128 increments were acquired in F1 consisting of 4 scans each.
Prior to Fourier Transformation, the data were apodized with a sine bell window function, linearly predicted to 512 data points and zerofilled in F1 to 1024 data points.

Selective Excitation Experiments were carried out in order to confirm the structure of the AZX+13+CN adduct.

A 1D selective ROESY experiment (Rotating frame Overhauser Effect Spectroscopy, selrogp, Bruker Biospin Ltd.) was carried out. The data was collected into 65k data points, over a spectral width of 12019 Hz, resulting in an acquisition time of 2.77 s. A relaxation delay of 2.4 s was employed, and a spin lock time of 100 ms.
Results

Structural characterisation of compound AZX

Test compounds often contain similar structural motifs to their metabolites, it is therefore common practice to first fully characterise the structure of the test/parent compound with accurate mass MSMS fragmentation. Characteristic fragment ions identified from the test compound MSMS can then be used to find and elucidate metabolite structures.

Analysis of AZX by LC-UV-MS/MSMS yielded a protonated molecular ion with an accurate mass of $[M+H]^+ 476.2243$ (+0.9 ppm). The proposed fragmentation pattern and LTQ-Orbitrap HCD MSMS spectrum of compound AZX (Figure 1) revealed 2 intense key fragments m/z 255.2175 (corresponding to the loss of AZX’s R-group) and m/z 141.1385 (corresponding to the homopiperazine isopropyl moiety).

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 combined to obtain a single pooled sample for each matrix. The sample is then analysed on a LC-UV-MSMS system in conjunction with a pre-dose sample spiked with analyte to obtain a final concentration of 10 μM (a concentration shown to produce a discernable UV response in the biological matrix).
Spiking of the pre-dose 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-MSMS in both, the 0-24 h rat urine-pooled sample and the AZX-spiked pre-dose urine sample. However, an unexpected major parent related peak was detected with a molecular ion of m/z 488. This observation was confirmed by re-spiking of parent compound into samples of fresh pre-dose/control urine (representing final AZX concentrations of 10 μM 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 approx. 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, as the molecular ion, i.e. the mass addition, could not be explained.

Therefore, in order 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 analysed in conjunction with repeat pre-dose and 0-24 h rat urine samples. A comparison of the UV chromatograms (extracted at λ=240-245 nm) for the AZX-spiked pre-dose urines (at 10 μM and 20 μM final concentrations) and the AZX solvent standards showed that in urine the UV-peak for AZX (RT= ca. 5.32min) was depleted whilst the m/z 488 product (RT= ca. 4.45min) was abundant (Figure 2). The AZX solvent standards and the dose solution,
however, 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 0-24 h rat blood, where the m/z 488 product was also observed, although at a reduced amount (at an approx. ratio of 40:60, m/z 488:AZX) as assessed by UV (data not shown). Furthermore, the spiking experiments with pre-dose blood, as conducted with the urine samples, again confirmed the formation of the m/z 488 product.

From this 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 0-24 h sample data (urine and blood) confirmed that this m/z 488 metabolite/product (RT= ca. 4.45min), represented the majority of the AZX related material in urine as determined by UV and mass spectrometry.

The m/z 488 product’s mass was equivalent to an increase of 12 Da from parent (AZX, [M+H]+ 476), although it represented an actual increase of 13 Da, when comparing molecular weights (as detailed in the following section).

**Structural characterisation of the novel metabolite/product, m/z 488 (AZX+13) by UPLC-MSMS.**

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 parent [M+H]+ 476.2243, suggesting addition of one carbon atom. This was further
substantiated following an elemental composition analysis where no rational alternative molecular formula could be identified.

The proposed fragmentation pattern and LTQ-Orbitrap HCD MSMS spectrum of this metabolite/product (Figure 3, RT=ca. 4.45min) 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 MSMS 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 Figure 3).

The difference between theoretical molecular weight and measured molecular ion of the proposed bridged homopiperazine.

The proposed bridged structure equates to the addition of 1 carbon and 1 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, as 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, whilst the calculated nominal mass of the parent (AZX) is 475 and the mass measured by +ESI mass spectrometry ([M+H]⁺) is 476 (Figure 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 MWs of AZX and AZX+13 results in a
mass difference of 13 Da, compared to the 12 Da difference in the measured molecular ions by mass spectrometry.

In order to highlight the +13 Da addition of this structurally unique product compared to 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 quarternary 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 quarternary nitrogen (i.e. the bridged homopiperazine moiety as shown in Figure 3), KCN was added to the AZX-spiked pre-dose and the pooled 0-24h urine sample. On addition of KCN, the AZX+13 product peak (m/z 488, RT= ca. 4.45min), reduced in size (based on its UV-response) whilst an additional peak [M+H]⁺ 515 (RT=ca. 5.85) was detected in each of the samples (Figure 4). Further investigation by MSMS 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.

The confirmation of the presence of the quarternary nitrogen on the homopiperazine led to the investigation of the mechanism of formation of AZX+13.
Investigation of the formation of AZX+13

Based on 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 1 carbon and 1 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 stabilised by forming the bridged homopiperazine (as shown in Figure 5).

To test this hypothesis, formaldehyde (10 μL, 37% in H2O) was spiked into the AZX test solution (10 μM, 500 μL) which was analysed 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 MSMS 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 in 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 $^1$H NMR Spectroscopy

Following identification by MS, the structures of the parent (AZX), the AZX+13 product and AZX+13+CN adduct were verified by $^1$H NMR spectroscopy (Figure 6A-C, respectively).

Following the addition of formaldehyde, no changes in the spectra were observed in the aromatic regions of AZX and AZX+13 spectra, indicating the site of modification being remote (data not shown).

The aliphatic region, however, highlighted several chemical shift changes supporting the proposed formation of the bridged homopiperazine (Figure 6A 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 ppm to 1.34 and 1.38 ppm. Similarly, all the residual homopiperazine protons experienced a shift to higher frequency. In addition to the chemical shift changes, two doublets (labelled Ha and Hb), not initially present in the parent spectrum, were observed at 4.68 ppm and 3.91 ppm, with a coupling constant of $^3J =$ 9.82Hz and an integral value of 1 proton per doublet, which showed a clear connectivity in the 2D COSY spectrum (data not shown).

The suggested structure is consistent with the proposed 1 carbon bridge across the homopiperazine.

Figure 6C shows the $^1$H 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 ppm and 3.5 ppm, which showed a strong connectivity in the 2D COSY spectrum (data not shown). Based on this evidence, 2 isomeric possibilities could be suggested (Figure 7, isomers 1 and 2). Selective irradiation experiments were employed to determine the correct structure of the AZX+13+CN adduct.

The selective ROESY experiment, irradiating the isopropyl methyl signals (labelled 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, (Figure 7). There was no enhancement of the bridged protons (labeled a and b), indicative of the 6-membered ring (isomer 2), hence providing evidence that this was not the preferred structure. Instead, the spectral data supported the presence of the NCH$_2$CN side chain (Isomer 1), following addition of KCN.
Discussion

This study demonstrated that compound AZX and various analogues containing a homopiperazine moiety reacted rapidly with formaldehyde in biological matrices to form a carbon and hydrogen addition bridged homopiperazine (+13 Da products). The origin/source of formaldehyde was, however, still unclear, as 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 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 1 carbon atom over their respective parent compounds), whilst our example, through a different mechanism, yielded a novel bridged homopiperazine with a quaternary nitrogen (following addition of 1 carbon and 1 hydrogen).

Hence, Yin et al. (2000) described the addition of +12 Da to parent compound identified in/derived from in vitro experiments where S9 fractions/hepatic incubations were spiked with an analyte in methanol. It was, however, concluded that the MeOH was first metabolised to formaldehyde (Teschke et al., 1974) which then reacted with a basic group on the incubated compound. Each of the tested compounds, containing either 1,2-
amino hydroxyl or 1,2-diamino reacted with the metabolic formaldehyde 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 2 molecules of 2,4-diaminotoluene with formaldehyde to give
bis(2,4diamino-5-toly1)methane.

Koeppel et al. (1991) described the formation of +12 Da products from the GC 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
GC-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 as 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 carried in order to rule out MeOH as the source of the formaldehyde or indeed as the reagent itself. The experiments aren’t described in detail here, however a brief summary is provided.

On spiking compound AZX/MeOH into ‘freshly collected’ pre-dose/control rat urine, utilising the MeOH:H₂O gradient, AZX+13 was produced spontaneously with 90-100% 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’ pre-dose/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 ca. 2 month prior to spiking, allowing degradation/evaporation of the formaldehyde, based on the half-life of formaldehyde (OECD/SIDS, 2002). After the 2 month ageing period, upon spiking of AZX, only ca. 5-10% 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-MSMS analysis was also carried out 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 90-100%), as assessed by UV quantification and MS analysis (data not shown). The AZX standards (the stock and test solutions, prepared in ACN), however, did not produce the product.
when subjected to UPLC-UV-MSMS analysis, ruling out ACN and formate as possible reagents.

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

The findings from our research appeared 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 ca. 0.1 mM concentration (Heck and Casanova, 2004). Formaldehyde and its oxidation product formate are reported to be key intermediates in the “1-carbon-pool” (Neuberger A., 1981). Certain xenobiotics such as MeOH, N-, O- or S-Me compounds and methylene chloride apparently can also contribute to this pool. The 1-C-pool is utilised 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, Toewsa 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. The reaction *in vitro*, however, relies on the presence of MeOH, e.g. used as spiking solvent, as a source of the formaldehyde (as outlined by Yin et al., 2000) and Cunningham et al., 1990).

Therefore, the potential for some basic xenobiotics to covalently bind to protein may not be flagged up by reactive metabolite screening or radiolabelled *in vitro* covalent binding studies.

Here, the Schiff base reactive intermediate, trapped due to the proximity of the 2 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.

As 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 favourable for the formation of a one-carbon bridge.

Various other homopiperazine analogues 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 that the homopiperazine moiety appears to be a highly reactive group, and the observations made in this study supported the decision to change the chemistry of these compounds.

In conclusion, this study has demonstrated that compound AZX and various analogues containing the homopiperazine moiety reacted rapidly with formaldehyde present in biological fluids, as a constituent of the 1-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 compounds’ potency while decreasing their reactivity. However, as we have shown in this study, this group is itself highly reactive with endogenous formaldehyde, forming bridged homopiperazines.

It is the suggestion of this paper that compound AZX and its homopiperazine analogues scavenge endogenous formaldehyde in vivo similar to aminoguanidine (AG) which was
reported by Kazachkov et al., 2007. What appears to be a desired effect with AG, 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 optimisation and pharmacokinetic assessments. Additionally this could artificially distort metabolite profiles in human metabolism studies, impacting (MIST)-investigations (FDA guidelines, 2008).

Overall, however, the study demonstrated that the bridged homopiperazine was readily formed in vivo, producing a stable product/metabolite, which didn’t 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 analytical 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
DMD #44917

References


OECD, SIDS, Formaldehyde. UNEP Publications. Human Health Hazards (issued in February 2002)

www.inchem.org/documents/sids/sids/FORMALDEHYDE.pdf


List of Legends

Figure 1. Proposed LTQ-orbitrap collision cell fragmentation of compound AZX (top) with accurate mass spectrum (bottom).

Figure 2: UV chromatograms of the AZX test solution (10 μM) (top) compared to a pre-dose rat urine sample, following spiking with AZX (10 μM) (bottom).

Figure 3: Proposed LTQ-orbitrap collision cell fragmentation of the postulated bridged homopiperazine (AZX+13) (top) with accurate spectrum (bottom).

Figure 4: The UV-chromatogram of the pre-dose urine following addition of KCN.

Figure 5: The proposed structure and formation of the bridged homopiperazine (AZX+13) through a quaternary Schiff base intermediate.

Figure 6: $^1$H NMR spectra of (A) the parent (AZX), (B) the AZX+13 product and (C) the CN-adduct (AZX+13+CN).

Figure 7: $^1$H NMR selective ROESY experiment (bottom) verifying the correct structure of the AZX+13+CN adduct as isomer 1 (top), following addition of KCN.
Figure 1

![Chemical structure diagram](image)

**[M+H]⁺ = 476.2238**
**MW = 475**

**m/z = 255.2179**
**MF=C₁₃H₂₇N₄O**

**m/z = 141.1386**
**MF=C₈H₁₇N₂**

**m/z = 112.1121**
**MF=C₇H₁₄N**

**m/z = 99.0917**
**MF=C₅H₁₁N₂**

**m/z = 86.0964**
**MF=C₅H₁₁N₂**

**m/z = 70.0651**
**MF=C₅H₁₂N**

**Relative Abundance Chart**

<table>
<thead>
<tr>
<th>m/z</th>
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<tbody>
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<td>255.2175</td>
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</tr>
<tr>
<td>141.1385</td>
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<td>99.0914</td>
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<td>86.0964</td>
<td></td>
</tr>
<tr>
<td>70.0651</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

RT: 5.32 min
AZX

RT: 4.45 min
AZX-product
Figure 3

Chemical structure and mass spectra data:

- **M+ = 488.2238**
- **MW = 488.2238**

Mass-to-charge ratios (m/z) and molecular formulas (MF):

- **m/z = 267.2179**  
  MF = C14H27N4O

- **m/z = 196.1444**  
  MF = C10H18N3O

- **m/z = 153.1386**  
  MF = C9H17N2

- **m/z = 98.0964**  
  MF = C6H12N

- **m/z = 82.0651**  
  MF = C5H8N

- **m/z = 110.0598**

- **m/z = 154.1451**

Relative abundance graph showing peaks at:

- **196.1443**
- **267.2178**
- **110.0598**
- **153.1384**
- **82.0651**
- **98.0961**
- **154.1451**

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Figure 5

Schiff base intermediate
Figure 6

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DMD Fast Forward. Published on May 1, 2012 as DOI: 10.1124/dmd.112.044917
Figure 7

Chemical structures showing the reaction of R1N+NO with KCN leading to two isomers: (isomer 1) and (isomer 2). The diagram includes a 1H NMR spectrum with peaks at 2.2', 3.3', and 9 ppm, indicating irradiation at Me-8.8'.