Amide Hydrolysis of a Novel Chemical Series of Microsomal Prostaglandin E Synthase-1 Inhibitors Induces Kidney Toxicity in the Rat

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ABSTRACT
A novel microsomal prostaglandin E synthase 1 (mPGES-1) inhibitor induced kidney injury at exposures representing less than 4 times the anticipated efficacious exposure in man during a 7-day toxicity study in rats. The findings consisted mainly of tubular lesions and the presence of crystalline material and increases in plasma urea and creatinine. In vitro and in vivo metabolic profiling generated a working hypothesis that a bis-sulfonamide metabolite (determined M1) formed by amide hydrolysis caused this toxicity. To test this hypothesis, rats were subjected to a 7-day study and were administered the suspected metabolite and two low-potency mPGES-1 inhibitor analogs, where amide hydrolysis was undetectable in rat hepatocyte experiments. The results suggested that compounds with a reduced propensity to undergo amide hydrolysis, thus having less ability to form M1, reduced the risk of inducing kidney toxicity. Rats treated with M1 alone showed no histopathologic change in the kidney, which was likely related to underexposure to M1. To circumvent rat kidney toxicity, we identified a potent mPGES-1 inhibitor with a low propensity for amide hydrolysis and superior rat pharmacokinetic properties. A subsequent 14-day rat toxicity study showed that this compound was associated with kidney toxicity at 42, but not 21, times the anticipated efficacious exposure in humans. In conclusion, by including metabolic profiling and exploratory rat toxicity studies, a new and active mPGES-1 inhibitor with improved margins to chemically induced kidney toxicity in rats has been identified.

Introduction
Deposition of drug-induced crystals within the kidneys can lead to acute and chronic kidney injury due to obstruction (Perazella, 1999; Yarlagadda and Perazella, 2008). A variety of drugs have been shown to precipitate in the renal tubules as a consequence of high local concentrations in the kidney and changes in drug solubility due to pH differences between urine and blood. One group of drugs that has been shown to cause kidney injury by crystalluria is the bacteriostatic antibiotic class of sulfonamides (Perazella, 1999). The sulfonamide drugs such as sulfadiazine and sulfamethoxazole are to a large extent excreted in the urine as parent molecules or metabolites. Because they are weak acids, they are relatively insoluble in acidic urine and have a tendency to precipitate in the tubular lumen when the pH of the urine drops below 5.5 (Hein et al., 1993; Perazella, 1999). High-dose sulfonamide treatment has been associated with an increased risk of sulfâ-crystal precipitation in the kidneys (Perazella, 1999).

A new chemical series of sulfonamide-containing compounds was recently identified as potential microsomal prostaglandin E2 synthase-1 (mPGES-1) inhibitors intended for pain treatment (Bylund et al., 2009). This series, which is composed of different bis-sulfonlamino derivatives, has two acidic pKa values around 2.5 and 10.5, CLogP values exceeding 2.5 and molecular weight exceeding 430 Da. Considering their molecular size and lipohilicity, parent compounds of this series and their conjugated metabolites are likely excreted into the bile (Yang et al., 2009), which differs from the antibiotic class of sulfonamides (Fleck et al., 1988; Perazella, 1999).

By assessing possible in vivo safety concerns in relation to anticipated efficacious exposures in humans already within the discovery program, drug candidates with a better chance of success in preclinical testing can be brought forward. mPGES-1 is an enzymatic drug target involved in the production of PGE2 (prostaglandin E2), a mediator of pain and inflammation (Samuelsson et al., 2007). It has been hypothesized that mPGES-1 inhibition might generate pain relieving anti-inflammatory effects similar to cyclooxygenase (COX) inhibitors such as conventional NSAIDs (nonsteroidal anti-inflammatory drugs) and COX-2 inhibitors (Samuelsson et al., 2007; Xu et al., 2008; Iyer et al., 2009). However, mPGES-1 inhibitors differ from COX inhibitors with respect to where in the biosynthetic cascade they inhibit PGE2 production. It has been hypothesized that mPGES-1 inhibitors might exhibit better safety profiles due to their propensity to affect the formation of other eicosanoids than PGE2 in a different way than conventional NSAIDs and COX-2 inhibitors (Samuelsson et al., 2007; Wang and FitzGerald 2010). However, this might also have

ABBREVIATIONS: AUC0–24, area under the curve for 24 hours; AUCτ, area under plasma concentration time curve needed for therapeutic effect; COX, cyclooxygenase; LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; mPGES-1, microsomal prostaglandin E synthase-1; NSAID, nonsteroidal anti-inflammatory drug; PGE2, prostaglandin E2; PK, pharmacokinetics; TK, toxicokinetics; WBA, whole blood assay.
predicted consequences for the translational aspects of PGE2 inhibition in pain relief (Wang and FitzGerald 2010).

NSAIDs and COX-2 inhibitors have previously shown good correlation between the average steady-state exposure in human subjects with a favorable analgesic effect and approximately 80% inhibition in a well established whole blood assay (WBA) that measures lipopolyasaccharide-induced COX2-mediated production of the biomarker PGE2 (Depré et al., 2000; Huntenburg et al., 2005; Huntenburg et al., 2006). The criterion for efficacious therapeutic concentration for this mPGES-1 inhibition project was based on an assumption that a steady-state concentration average corresponding to 10 times the WBA IC50 value would be satisfactory for pain treatment. However, it is presently unknown whether mPGES-1 inhibitors are efficacious for pain treatment in humans and whether the WBA can be used as a translational tool for this novel group of drugs.

In an attempt to develop a novel mPGES-1 inhibitor, we experienced problems associated with chemically induced kidney toxicity in the rat at exposures representing less than 4 times the anticipated efficacious exposure in man. The aim of this study is to describe problem-solving strategies in a drug discovery project encumbered with kidney toxicity. More specifically, we describe the toxicologic kidney findings observed in a 7-day toxicity study, the establishment of a hypothesis for toxicity origination, and the discovery of a new inhibitor with a reduced kidney toxicity risk profile.

Materials and Methods

Chemicals and Reagents. Test compounds, including the metabolites referred to as M1 and Hyd, originated from AstraZeneca’s compound collection (Södertälje, Sweden), and the synthesis of such derivatives has been described in a patent (Bylund et al., 2009). Chemical structures of AZ 7847, AZ’4270, AZ’4284, AZ’0908, and M1 are shown in Fig. 1. Those labeled [14C]-AZ 7847 and [13C]-AZ 4270 were synthesized by the isotope chemistry laboratory at AstraZeneca, Södertälje, Sweden, with a specific activity of 2.1 GBq/mmol and a radiochemical purity exceeding 99.4%. The 13C label was located, for both compounds, on the carboxyl carbon as depicted in Fig. 5. All other chemicals used were of analytical grade and obtained from commercial suppliers.

Solubility Measurements and pKa Predictions. Solubility samples in rat urine (pH 7.0) were incubated at 37°C for 5 hours and analyzed using a Hewlett Packard 1100 system (Agilent Technologies, Santa Clara, CA) equipped with a Symmetry C18 column (3.5 μm, 4.6 x 50 mm), a photodiode array detector, and a mass single detector. The mobile phases consisted of water with 0.3% acetonitrile. The mobile phase was a binary mixture of 0.1% formic acid/water (solvent A) and methanol gradient were used. For quantification of the M1 metabolite, the mass spectrometer was set to select the [M-H]− precursor ion at mass-to-charge ratio 234.9 and for the product ion at mass-to-charge ratio 171.1. Before the LC-MS/MS analysis, the samples were precipitated with acetonitrile.

The LC-MS analysis for metabolic profiling was performed as described elsewhere (Sohlenius-Sternbeck et al., 2010). Briefly, hepatocytes at concentrations 1 x 10⁶ cells/ml in William’s medium were incubated at 37°C with gentle shaking in a 5% CO₂ atmosphere at substrate concentrations of either 1 μM or 10 μM. All incubations were stopped by the addition of 2 volumes of ice-cold acetonitrile and were then centrifuged before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The metabolic capacity of hepatocytes was checked in-house using a set of probe substrates (Floby et al., 2009).

Liquid Chromatography Mass Spectrometry Analysis. Liquid chromatography mass spectrometry (LC-MS) analysis for quantifications was performed with a Micromass Quattro Micro triple quadrupole (Waters Corporation, Milford, MA), as previously described elsewhere (Briem et al., 2007) with the exception of the analysis of the M1 metabolite. Total concentration of M1 in plasma was determined by LC-MS/MS using electrospray in the negative ionization mode. Because the analyte was not retained on a conventional C18 column, a porous graphitic carbon column and methanol gradient were used. For quantification of the M1 metabolite, the mass spectrometer was set to select the [M-H]− precursor ion at mass-to-charge ratio 234.9 and for the product ion at mass-to-charge ratio 171.1. Before the LC-MS/MS analysis, the samples were precipitated with acetonitrile.

Animals. Wistar Hannover rats, approximately 8 weeks old, were used for toxicity studies, and male Sprague-Dawley rats were used for pharmacokinetic (PK) studies. All rats were obtained from Taconic M&B A/S (Ry, Denmark). The rats were randomized into groups (n = 2–4/sex) by body weight and were acclimatized for approximately 2 weeks before the start of the study. The animals were housed up to 3 per cage in transparent plastic cages. The bedding material consisted of aspen wood chips. Plastic tunnels and aspen chew blocks were also provided. The animals had free access to pelleted RMI (E) SQC diet and water from the site drinking water supply, except during urine collection when access to food was temporarily withdrawn. The animal room was illuminated by artificial light from fluorescent tubes on a 12-hour light/dark cycle. The temperature and relative humidity were 17° to 23°C and 40 to 70%, respectively. All experiments were approved by the local animal ethics
for toxicokinetic analysis (Jonsson et al., 2012). The TK parameters study 2. In toxicity study 3, a microsampling technique was used to sample data but both the parent molecules and M1 metabolite were analyzed in toxicity samples were analyzed for only the parent molecule in toxicity studies 1 and 3, minutes at +4°C for 10 minutes at 1500 samples were immediately placed on ice, and they were centrifuged within 30 blood was collected in EDTA Microtainer tubes (BD, Plymouth, UK). The blood study 1 and on the last day of administration. For satellite animals in toxicity exposure analysis only) were used for toxicokinetic (TK) analysis per dose group in the second study with AZ and AZ in the first study, but only 2 satellite female rats were used per dose group in the third toxicity study, five animals per dose group of AZ was formulated as solutions in 20% (w/v) polyethylene glycol 400 in 0.2 M meglumine (vehicle 1); the M1 metabolite was formulated a solution in 1% (w/v) hydroxypropyl methylcel- (v/v) polyethylene glycol 400 in 0.2 M meglumine (vehicle 1); the M1 metabolite was formulated a solution in 1% (w/v) hydroxypropyl methylcel- lulose and 0.1% (w/v) Tween 80 (vehicle 2); and AZ was formulated as solution in 15% (w/v) hydroxypropyl-b-cyclodextrin in 0.2 M meglumine (vehicle 3). Animals used for toxicity studies were dosed once daily by oral gavage (10 ml/kg body weight) for 7 or 14 days. Control animals were treated with either vehicle 1 or vehicle 2. Doses were set based on low-dose PK studies where linear kinetics was assumed.

Toxicokinetics. Two male and two female satellite rats (animals used for exposure analysis only) were used for toxicokinetic (TK) analysis per dose group in the first study, but only 2 satellite female rats were used per dose group in the second study with AZ and AZ, and AZ and AZ and the M1 metabolite. In the third toxicity study, five animals per dose group of AZ were used for TK analysis. All compounds were dosed once daily for 7 consecutive days, and blood samples were taken at 2, 4, 6, 7, and 24 hours on day 1 and on the last day of administration. For satellite animals in toxicity studies 1 and 2, approximately 0.3 ml of blood was taken via the tail vein and was collected in EDTA Microtainer tubes (BD, Plymouth, UK). The blood samples were immediately placed on ice, and they were centrifuged within 30 minutes at +4°C for 10 minutes at 1500 g to obtain plasma. The plasma was transferred to 1.4 ml Matrix vials and was immediately frozen to −70°C. The samples were analyzed for only the parent molecule in toxicity studies 1 and 3, but both the parent molecules and M1 metabolite were analyzed in toxicity study 2. In toxicity study 3, a microsampling technique was used to sample data for toxicokinetic analysis (Jonsson et al., 2012). The TK parameters C and area under the curve for 24 hours (AUC) were calculated with Pharsight WinNonlin (Pharsight, St. Louis, MO). All TK values are presented as mean values from at least two animals. Low-dose PK studies for dose setting in toxicity studies were performed as described elsewhere (Briem et al., 2007). Clinical Pathology. Blood plasma samples (into tubes with lithium heparin) were taken from the orbital plexus of animals anesthetized with isoflurane and oxygen at the end of the dosing period; they were analyzed for urea and creatinine. The detection of urea and creatinine levels was performed on a clinical chemistry analyzer COBAS 6000 e501 (instrument and reagents by Roche, Penzberg, Germany). The limit of detection was 0.5 mmol/l and 5 μmol/l, respectively. Individual urine samples from animals placed in metabolism cages were collected on ice for about 6 hours starting immediately after dosing and were analyzed for crystals by cystologic examination. Histopathology. The rats were killed by exsanguination from a common carotid artery under isoflurane and oxygen anesthesia 1 day after the final dose was given. The body weight was recorded, and the kidneys were removed, fixed, and preserved in buffered formalin. The preserved kidney specimens were trimmed, processed through graded alcohol and clearing agent, infiltrated, and embedded in paraffin, sectioned (5 μM), and stained with hematoxylin and eosin (Bancroft and Gamble, 2008). The sections were examined for histopathologic changes using a light microscope.

### Results

**Kidney Toxicity of AZ 7847.** Estimations of efficacious therapeutic concentration in humans (AUCther) were based on an assumption that an average steady-state concentration corresponding to 10 times the attained IC value (unpublished data) in a well established whole blood PGE2 assay (Huntjens et al., 2005) would be satisfactory for pain treatment. Doses aiming to achieve 10 and 20 times the AUCther

### Table 1

Toxicity study design

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Sex and No. of Animals</th>
<th>Comments on TK Samples</th>
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<tr>
<td>AZ 7847 500</td>
<td>2M + 2F</td>
<td>2M + 2F satellite animals</td>
</tr>
<tr>
<td>AZ 7847 1000</td>
<td>2M + 2F</td>
<td>2M + 2F satellite animals</td>
</tr>
<tr>
<td>Vehicle 1</td>
<td>0</td>
<td>4F</td>
</tr>
<tr>
<td>Vehicle 2</td>
<td>0</td>
<td>4F</td>
</tr>
<tr>
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<td>0</td>
<td>4F</td>
</tr>
<tr>
<td>MI 500</td>
<td>0</td>
<td>4F</td>
</tr>
<tr>
<td>MI 2000</td>
<td>0</td>
<td>4F</td>
</tr>
<tr>
<td>AZ 4270 170</td>
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<td>4F</td>
</tr>
<tr>
<td>AZ 4284 76</td>
<td>0</td>
<td>4F</td>
</tr>
<tr>
<td>AZ 0908 435</td>
<td>0</td>
<td>4F</td>
</tr>
<tr>
<td>AZ 0908 900</td>
<td>0</td>
<td>4F</td>
</tr>
</tbody>
</table>

F, female; M, male; TK, toxicokinetic.

* Same vehicle as AZ 7847, AZ 4270, and AZ 4284.

* Same vehicle as for M1.

### Table 2

Toxicokinetic summary of AZ 7847 in toxicity study 1

<table>
<thead>
<tr>
<th>Dose (μmol/kg)</th>
<th>Sex and No. of Animals</th>
<th>Anticipated AUC (μMh)</th>
<th>Day 1</th>
<th>Day 7</th>
<th>AUC0–24/AUCmax (ratio)</th>
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</table>
in humans were used. Rats were orally administered either 500 or 1000 μmol/kg per day for 7 consecutive days. LC-MS and TK analyses revealed that a lower than anticipated exposure on day 7 for low-dose animals was achieved and that no obvious sex difference in AUC was detected (Table 2). No high-dose exposure data at day 7 were available due to premature death or sacrifice.

Toxicologic evaluation showed severe kidney injury in animals from the high-dose group. Necropsy findings included increased kidney weights and pale kidney discoloration. The histopathologic evaluation showed marked tubular degeneration, characterized by the presence of dilated tubules with cellular remnants within the lumen, mixed with basophilic, dilated tubules with hypertrophic nuclei and cytoplasm. The distribution of this finding was cortex and medulla, with emphasis on the medulla. Additional changes in the kidney included multiple pyogranuloma (accumulation of neutrophils/macrophages surrounding amorphous/crystalline material within tubules in the cortex and medulla), inflammation and hemorrhage in the renal pelvis, free crystals in the renal pelvis, transitional cell hyperplasia, and papillitis.

Figure 2 shows the histopathology comparison of a kidney from a control rat and from the kidney of a rat treated with 1000 μmol/kg AZ’7847 per day for 7 days. Analysis of plasma showed extremely high levels of urea and creatinine in rats treated with 1000 μmol/kg per day for 7 days (Fig. 3). At the low dose, 500 μmol/kg, moderate tubular degeneration/regeneration was observed. The characteristic kidney damage and identification of crystal deposits suggested that kidney toxicity was caused by tubular precipitation of AZ’7847-associated drug material.

**Amide Hydrolysis of AZ’7847.** Metabolic profiling in rat hepatocytes showed that amide hydrolysis of AZ’7847 was a major metabolic pathway in vitro. Comparison of chromatograms detected by mass spectrometry (Fig. 4A) and radiodetection (Fig. 4B) showed that the carboxylic acid metabolite formed (depicted as Hyd in Fig. 5) had a much lower mass spectrometry response than the parent compound, whereas the corresponding bis-sulfonamide metabolite (depicted as M1 in Fig. 5) was not detected by either MS analysis or radiodetection. That amide hydrolysis was a major metabolic pathway in vivo was confirmed by metabolic profiling of the plasma samples from the toxicity study (Fig. 4C). Additional major metabolites tentatively identified by accurate mass LC-MS analysis were a mono-oxidized metabolite (marked +16), a combined oxidized and sulfate-conjugated metabolite

![Figure 2: Histopathologic changes in the rat kidney papilla. (A) Control animal treated with the same formulation as AZ’7847 for the same time period. (B) Distinct kidney necrosis, inflammation, and crystal deposits were detected in the rats treated with AZ’7847. Kidneys stained with hematoxylin and eosin; magnification, 100×.](image)

![Figure 3: Kidney functional biomarkers. (A) Urea. (B) Creatinine. Mean values generated from two male and two female rats. The standard error of the mean is shown in graphs.](image)
(marked +16+Sulf), a metabolite likely formed by amide hydrolysis in combination with mono-oxidation (Hyd+16), and a metabolite formed by amide hydrolysis in combination with mono-oxidation and sulfate-conjugation (Hyd+16+Sulf). Metabolic in vitro profiling in both cryopreserved and freshly prepared human hepatocytes and cryopreserved dog hepatocytes revealed no indication that AZ'7847 had undergone amide hydrolysis. Metabolic profiling experiments in human hepatocytes suggested that AZ'7847 is highly metabolically stable (Fig. 4D).

The detection of large quantities of the Hyd metabolite in rats obviously suggested formation of large amounts of the corresponding M1 metabolite (Fig. 5). Measurements of the M1 solubility in rat urine at pH 7.0 revealed a moderate solubility of 83 μM, which was similar to the parent molecule (45 μM). The acidic pKa of the M1 metabolite was predicted to be approximately 9.5. Based on the detection of this M1 metabolite that structurally resembles sulfonamide-containing antibacterial agents and the characteristic kidney damage, we hypothesized that a compound with a low degree of hydrolysis would be superior with respect to kidney toxicity risk.

**Evaluation of Kidney Toxicity Hypothesis.** Two compounds, AZ’4270 and AZ’4284 (Fig. 1), containing structural modifications intended to block amide hydrolysis by steric hindrance were identified. No hydrolysis in rat hepatocytes (Fig. 6, A and B) was detected for either of the two compounds, and they were chosen for an exploratory rat toxicity study. Both compounds exhibited IC50 values above 2 μM in the human WBA (unpublished data) and were therefore only considered as tool compounds. A 7-day rat toxicity study focusing on kidney toxicity and M1 exposure was performed with the M1 metabolite and the compounds AZ’4270 and AZ’4284 together with AZ’7847 as a positive control (toxicity study 2 in Table 1). Doses were set to achieve a similar and a significantly higher exposure than what had been achieved by 1000 μmol/kg AZ’7847 in the previous study. LC-MS and TK analyses showed that the exposures of AZ’4270 and AZ’4284 per dose unit were more than 5 times higher than for AZ’7847 (Table 3). TK analysis also showed that the M1 metabolite in the plasma samples of rats administered AZ’4270 and AZ’4284 was much lower than with the positive control AZ’7847. The ratio between the exposure of the M1 metabolite and

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**Fig. 4.** Metabolic profiling of AZ’7847. (A) Rat hepatocyte metabolites detected with LC-MS. (B) Rat hepatocyte metabolites detected with radio monitoring (same LC chromatogram as in (A)). (C) In vivo rat metabolism detected with LC-MS. Plasma sample used for analysis originated from a low-dose (500 μmol/kg per day) rat at 4 hours on day 7. (D) Freshly prepared human hepatocytes analyzed with LC-MS. The MS chromatograms are showing the combined base peak intensities of the molecular ion [M-H]- of the parent molecule m/z 439.042 and the potential metabolites with m/z values 221.060, 237.055, 317.012, 397.092, 453.021, 455.037, 471.032, 473.048, 493.048, 534.994, 631.069, and 762.121. Putative peak identification was suggested by LC-MS analysis with accurate mass determination. The Hyd metabolite identification was confirmed by comparison of MS/MS spectra and LC retention time generated with a synthesized standard.
the exposure of the parent molecule at day 7 was more than 30 times lower for AZ’4270 and more than 75 times lower for AZ’4284 as compared with AZ’7847. The exposure of M1 after M1 administration was significantly lower than for the positive control AZ’7847, and approximately the same exposure was achieved after the administration of 500 and 2000 μmol/kg per day (Table 3). M1 accumulation appeared to be low during the administration period, except in rats treated with AZ’7847 in whom both AUC₀-2₄ and Cₘₐₓ increased more than 3-fold.

The histopathologic analysis of kidneys from rats treated with AZ’7847 was similar to the previous study, and elevated levels of the kidney functional biomarkers urea and creatinine were detected (not shown). Neither pathophysiological findings nor elevation of kidney functional biomarkers were detected for AZ’4270 and AZ’4284 despite exposure levels of parent molecules in the same range or higher than AZ’7847. However, crystals were seen in the urine of animals treated with AZ’4270 (only in high dose) but not with AZ’4284. No histopathologic findings or changes in creatinine and urea parameters were detected for rats treated with the M1 metabolite. Crystals were observed in the urine from both dose groups of animals dosed with the M1 metabolite.

Identification and Evaluation of AZ’0908. We identified a novel and potent mPGES-1 inhibitor AZ’0908 (Fig. 1), which exhibited no detectable amide hydrolysis in rat hepatocytes (not shown). A rat PK study using a single dose of 10 μmol/kg (Fig. 7) suggested that the rat exposure per dose unit of AZ’0908 and the ratio of the plasma concentration of the M1 metabolite and the parent molecule were drastically better than for AZ’7847 (Table 4). Considering the improved rat PK and possibly also improved redirection of metabolism, AZ’0908 was chosen for evaluation in a 14-day rat study focusing on kidney toxicity (toxicity study 3 in Table 1).

Doses were set to achieve approximately 20-fold and 40-fold margins to anticipated AUCₜₚₑₜ in humans. Samples for TK analysis were taken by microsampling, and the M1 metabolite was not monitored in the study. TK analysis showed that the exposure objectives were achieved and that the two doses exhibited dose-proportionality for AUC₀–₄ but not for Cₘₐₓ (Table 3). Toxicologic evaluation showed that the low dose, which generated exposures of 21 times the anticipated AUCₜₚₑₜ in humans, was not associated with any significant kidney toxicology. However, for 6 out of 10 animals in the high-dose group tubular degeneration was detected, and in two of these animals moderate or severe crystalline deposits were observed. Also, creatinine and urea plasma levels were substantially increased at approximately the same levels as for AZ’7847. However, there were no changes in plasma chemistry parameters in the low-dose group with AZ’0908. Crystals in the urine were found in both dose groups.

![Fig. 5. Amide hydrolysis of AZ’7847. Amide hydrolysis results in the formation of two metabolites: M1 and Hyd. The position of the [¹⁴C]-labeling is depicted in the structure.](image)

![Fig. 6. Metabolic profiling of AZ’4270 and AZ’4284 in rat hepatocytes. (A) LC-MS chromatogram of a rat hepatocyte incubation of AZ’4270. The chromatogram is showing the combined base peak intensities of the molecular ion [M-H]⁻ of the parent molecule m/z (mass-to-charge ratio) 517.071 and the potential metabolites with m/z values of 297.073, 299.089, 315.084, 395.041, 475.121, 515.055, 531.050, 533.066, 549.061, 571.073, 613.022, 709.098, 838.135, and 840.129. The metabolic profiling was confirmed by radio monitoring detection of [¹⁴C]-AZ’4270 (not shown). (B) LC-MS chromatogram of a rat hepatocyte incubation of AZ’4284. The chromatogram is showing the combined base peak intensities of the molecular ion [M-H]⁻ of the parent molecule m/z 433.034 and the potential metabolites with m/z values of 213.036, 215.052, 231.047, 311.003, 391.0084, 419.018, 431.018, 433.034, 435.013, 435.049, 447.013, 449.028, 465.028, 467.039, 487.035, 528.985, and 625.061. Putative peak identification was suggested by LC-MS analysis with accurate mass determination. P = parent molecule, and differences in m/z value in relation to parent m/z value are shown for major peaks.](image)
### TABLE 3
Toxicokinetic summary of toxicity study 2

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</table>

### Discussion

This study is an example of problem solving in a drug project encumbered with kidney toxicity in rats. The mPGES-1 inhibitor AZ’7847 intended for pain treatment was assessed in a 7-day toxicity study in rats and was found to induce kidney injury at exposures representing less than 4 times the anticipated efficacious exposure in humans. Because AZ’7847 exhibited no activity on the rat mPGES-1 target (not shown), it was unlikely that the observed toxicity was related to target activity. Instead, the characteristic kidney damage and the identification of crystal deposits suggested that the kidney toxicity more likely was caused by tubular precipitation of AZ’7847-associated drug material. Based on metabolic profiling experiments, we hypothesized that the crystal deposits were composed of a bis-sulfonamide metabolite (M1) associated with amide hydrolysis. To test the hypothesis, rats were given the M1 metabolite and two low potency mPGES-1 inhibitor analogs, where amide hydrolysis was undetectable in rat hepatocyte experiments. The results suggested that compounds with a reduced propensity to undergo amide hydrolysis and thus having less ability to form M1 reduced the risk of inducing kidney toxicity. Additional support for our hypothesis of M1 induced kidney toxicity can be found in a recent methodology study (Nilsson et al., 2012) where it was confirmed that the crystal deposits in rat kidneys formed after AZ’7847 administration were composed of the M1 metabolite. This was realized by using nuclear magnetic resonance analysis and matrix-assisted laser desorption ionization mass spectrometry imaging.

Rats treated with M1 alone surprisingly showed no histopathologic changes in the kidney. However, TK analysis revealed low exposure levels and non-dose-proportionate kinetics for the M1 metabolite. This was likely related to absorption limitations, as the solubility of the M1 metabolites was poor. The lack of kidney toxicity in rats treated with the M1 metabolite alone was likely due to underexposure. It has been shown that C<sub>max</sub> is important for the development of kidney injury associated with drug precipitation for acyclovir, as rapid bolus injections lead to an increased risk of acute renal failure as compared with slow infusions of the same dose (Gnann et al., 1983). However, in this case, when the M1 metabolite is released from the parent molecule, the M1 C<sub>max</sub> appears to be directly related to its exposure. The threshold for M1-induced rat kidney injury appeared to be rather high. Exposure to M1 at approximately 1300 μMh and C<sub>max</sub> of 80 μM generated visual crystals in the urine, but neither changes in kidney functional biomarkers nor detectable histopathologic changes occurred. A comparison of M1 exposure between day 1 and day 7 clearly showed an accumulation over time in rats treated with AZ’7847. In rats treated with M1 alone, AZ’4270 or AZ’4284, accumulation was significantly less or not obvious. This is likely related to severely
decreased kidney function in the rats that received 1000 μmol/kg AZ’7847.

Despite the lack of detection of amide hydrolysis by their corresponding carboxylic acids in vitro, the tool compounds AZ’4270 and AZ’4284 underwent, to some extent, amide hydrolysis in vivo as judged by the detection of the corresponding M1 metabolite in plasma. Amide hydrolysis can be catalyzed by several different hydrolases in different tissues (Testa and Krämer, 2007). It is possible that amide hydrolysis in vivo of AZ’4270 and AZ’4284 to a large extent was carried out by extrahepatically expressed enzymes in the rat. Nevertheless, there appeared to be a correlation between the degree of hydrolysis in vivo and the propensity to undergo amide hydrolysis in rat hepatocytes in vitro. Interestingly, amide hydrolysis might be for the most part a rat-specific phenomenon. In human and dog hepatocytes, amide hydrolysis could not be detected for AZ’7847, as its corresponding carboxylic acid metabolite (Hyd). In addition, human hepatocyte experiments suggested that the compound AZ’7847 was more stable in vitro when compared with rats. It is therefore possible that M1-induced kidney toxicity after treatment of mPGES-1 inhibitors of this chemical series would not pose a major threat in humans because amide hydrolysis occurrence is likely lower and the doses needed to induce toxicity would be unattainable for all practical purposes. However, identification of a rodent toxicity species would be essential for progression into clinical trials; therefore, an extensive effort was conducted to identify an mPGES-1 inhibitor candidate that could achieve larger margins in the rat.

A novel and potent mPGES-1 inhibitor, AZ’0908, with a low affinity for amide hydrolysis and thus improved rat PK was identified. This was likely achieved by structural modifications, which resulted in affinity for amide hydrolysis and thus improved rat PK was identified. Based on the expression data, rat hepatocytes were used as a model system to study the metabolism of AZ’0908 and its corresponding carboxylated acid metabolite (Hyd). In addition, human hepatocyte experiments suggested that the compound AZ’7847 was more stable in vitro when compared with rats. It is therefore possible that M1-induced kidney toxicity after treatment of mPGES-1 inhibitors of this chemical series would not pose a major threat in humans because amide hydrolysis occurrence is likely lower and the doses needed to induce toxicity would be unattainable for all practical purposes. However, identification of a rodent toxicity species would be essential for progression into clinical trials; therefore, an extensive effort was conducted to identify an mPGES-1 inhibitor candidate that could achieve larger margins in the rat.

A novel and potent mPGES-1 inhibitor, AZ’0908, with a low affinity for amide hydrolysis and thus improved rat PK was identified. This was likely achieved by structural modifications, which resulted in reduced amide hydrolysis. In a 14-day rat toxicity study, AZ’0908 exhibited a margin that was 21-fold the anticipated AUC_th in human. As a result of plasma deficiency due to microsampling, the M1 metabolite was not monitored in this study. However, for AZ’0908 or any other compound of this chemical series to progress into clinical trials, margins based on exposures of both the parent molecule and the M1 metabolite would have to be established in future toxicity studies. Although in vitro results indicate that the M1 formation is less pronounced in humans than in rats, uncertainties exist concerning the intraspecies translational aspects of M1 formation. Therefore, future clinical trials that include compounds of this chemical series would need to exercise caution regarding the initial administration of parent compound and its exposure in relation to M1.

In conclusion, we have identified a novel mPGES-1 inhibitor that can be toxicologically evaluated in the rat. This was achieved by exploratory studies that linked together aspects of both drug metabolism and toxicity.

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