ABSTRACT:

Pioglitazone is in the class of compounds known as the thiazolidinediones and is used to treat type 2 diabetes mellitus. The first in its class compound, troglitazone, was withdrawn from the U.S. market in 2000 due to a high incidence of hepatotoxicity and drug-induced liver failure. Reactive ring-opened products of troglitazone have been identified and evidence suggests that these reactive intermediates might be a potential cause of hepatotoxicity. The present work shows that pioglitazone has a reactive ring-opened product which was trapped by glutathione and positively identified by high performance liquid chromatography with tandem mass spectrometry accurate mass measurements. The novel thiazolidinedione ring-opened products of pioglitazone were identified in rat and human liver microsomes and in freshly isolated rat but not human hepatocytes.

Pioglitazone is in the class of compounds known as the thiazolidinediones (troglitazone and rosiglitazone are in this class) and is used to treat type 2 diabetes mellitus by targeting the peroxisome proliferator-activated receptor-γ. Pioglitazone is metabolized in the liver primarily by cytochromes P450 3A4 and 2C8 (Actos Package Insert, Takeda Pharmaceuticals America, Inc., Lincolnshire, IL and Eli Lilly and Company, Indianapolis, IN July 2002 (http://www.fda.gov/cder/foi/label/1999/21073rbl.pdf); Sahi et al., 2003) to several oxidative metabolites (Krieter et al., 1994; Sohda, 1995; Tanis et al., 1996; Kiyota et al., 1997; Eckland, 2000; Shen et al., 2003). There have been extensive reports published on the hepatotoxic effect of troglitazone that led to its removal from the U.S. market in 2000 (Gitlin et al., 1998; Neuschwander-Tetri et al., 1998; Watkins and Whitcomb, 1998; Faich and Moseley, 2001). Evidence suggests that a potential cause of hepatotoxicity by troglitazone might be the formation of a reactive metabolite that could bind to cellular protein or DNA (Kassahun et al., 2001; Smith, 2003; Tolman and Chandramouli, 2003). The glitazones (Fig. 1) all have a thiazolidinedione ring, and at least one of the metabolites of troglitazone, pioglitazone, and rosiglitazone involves a ring-opened product of the thiazolidinedione ring (Kassahun et al., 2001; Shen et al., 2003; Alvarez-Sanchez and Paechler, 2004). However, the ring-opened product of pioglitazone has only been identified in dog liver microsomes. The ring-opened products for rosiglitazone (Avandia), currently the number one prescribed medicine in its class, and pioglitazone have yet to be discovered or reported as being formed in human in vitro systems or in the clinic. Due to recent findings of liver injury in patients undergoing pioglitazone treatment (Maeda, 2001; Chase and Yarze, 2002; May et al., 2002), pioglitazone was evaluated in rat and human liver microsomes and suspensions of freshly isolated hepatocytes to determine whether a reactive ring-opened product would be formed. To accomplish this evaluation, a generic method for trapping reactive electrophilic metabolites was used (Chen et al., 2001). In this method, the reactive intermediate is trapped using excess glutathione (GSH), and the trapped adduct is detected using the characteristic 129-amu neutral loss from GSH. This method offers an advantage of having the potential to trap and detect all species reactive with GSH and providing molecular weight information. The molecular weight information was used to further identify metabolites by quadrupole time of flight (Q-ToF) accurate mass measurements.

Materials and Methods

Materials. Pioglitazone was synthesized by the Medicinal Chemistry department at GlaxoSmithKline. The structure of pioglitazone is shown in Fig. 1 (mol. wt. = 356.45). Potassium phosphate monobasic (KH₂PO₄) and potassium phosphate dibasic trihydrate (K₂HPO₄·3H₂O) were purchased from J. T.

ABBREVIATIONS: GSH, reduced glutathione; amu, atomic mass unit(s); LC/MS, high performance liquid chromatography with tandem mass spectrometry; Q-ToF, quadrupole time of flight; HLM, human liver microsome; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; MRM, multiple reaction monitoring.
Baker (Phillysburg, NJ). Magnesium chloride (MgCl₂), β-nicotinamide adenine dinucleotide phosphate, reduced (NADPH), dexamethasone, Percoll, and GSH were purchased from Sigma-Aldrich (St. Louis, MO). Rat liver microsomes and human liver microsomes (HLMs) were purchased from Xenotech, LLC (Lenexa, KS). Human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD) and Xenotech, LLC, and rat hepatocytes were isolated at GlaxoSmithKline from male Sprague-Dawley rats purchased from Charles River Breeding Laboratories (Portage, MI) using the method described below. Fetal bovine serum and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from LGC Promochem, Inc. (Carlsbad, CA) and ITS-H11001 MD. Penicillin, streptomycin, and insulin were purchased from Invitrogen (Carlsbad, CA) and ITS+ culture supplement (625 μg/ml insulin, 625 μg/ml transferrin, 625 ng/ml selenium, and 100 mg/ml albumin) was purchased from BD Biosciences Discovery Labware (Bedford, MA).

Microsomal Incubation. Reagents were prepared by dissolving solids or diluting liquids in potassium phosphate buffer (50 mM) at concentrations shown below. A 10 mM stock solution of pioglitazone was prepared in dimethyl sulfoxide (DMSO). Each incubation vial contained substrate (100 μM pioglitazone final concentration, 1% DMSO in incubate), rat liver microsomes or HLMs-Buffer (345 μl of a 20 mg/ml stock preparation, 2 mg/ml liver microsomes), GSH (10 mM final concentration, no GSH controls received 100 μl of buffer), and cofactor (2 mM NADPH and 5 mM MgCl₂ final concentration, no cofactor controls received 50 μl buffer) in this order. Three separate control incubations were performed: incubation in the absence of substrate, glutathione, or cofactor. The final incubation volume was 500 μl for the glutathione-trapping experiment and scaled up to 5 ml for LC/MS accurate mass measurement. Samples were incubated at 37°C for 30 min and then quenched with 2% trifluoroacetic acid (500 μl) or 1% trifluoroacetic acid.

Hepatocyte Incubation. For incubations, rat and human hepatocytes were diluted in serum-free DMEM (supplemented as described above) to 1.0 × 10⁶ cells/ml, and 0.5 ml was added per well in 12-well plates. Hepatocytes were then placed on a rotary shaker at 70 rpm in a humidified incubator (95:5% air/CO₂) maintained at 37°C. After 10 min in the incubator, hepatocytes were dosed with 0.5 ml of pioglitazone at 20 μM and placed back in the incubator. The final concentration of drug studied was 10 μM in a total incubation volume of 1 ml (prepared to maintain <0.1% final DMSO concentration). At 1, 2, and 4 h, the reaction was terminated by addition of acetonitrile and ethanol (80:20, 2 ml) with 1% acetic acid. An initial time point (t₀) was prepared by adding diluted hepatocytes and drug to a well already containing the organic solvent mixture. Precipitated protein was removed by centrifugation and the resultant supernatant was prepared for subsequent LC/MS analysis. Troglitazone was used as a positive GSH adduct control for cell viability.

LC/MS with Neutral Loss Scanning. Samples from microsomal and hepatocyte incubations (1 ml) were prepared for analysis using a solid phase extraction 96-well plate (10-mg Oasis mixed-mode cation exchange; Waters, Milford, MA) preconditioned with 1 ml of methanol and 1 ml of 2% trifluoroacetic acid. The wells were washed with 0.1 M hydrochloric acid (1 ml) followed by methanol (0.3 ml), and samples were eluted with 50 mM ammonium hydroxide in methanol (0.5 ml). Sample separation was achieved using a Hewlett-Packard 1100 HPLC system (Hewlett Packard, Palo Alto, CA) with gradient capabilities. A Waters Atlantis column (dC18, 3-μm, 2.1 × 50 mm) was used with a sample injection volume of 50 μl. The mobile phase system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient conditions were 5% B (0–1 min), 5 to 95% B (1–5 min), and then back to initial conditions at 5.6 min. Samples were analyzed using an Applied Biosystems/MDS Sciex (Foster City, CA) API4000 in positive TurbolonSpray mode. The detection method was a generic neutral loss method of 129 amu, which will detect any ion that has a neutral loss of 129 in a predetermined mass range (Chen et al., 2001). Parameters for the mass spectrometer were set as follows: curtain gas 10 psi, temperature 600°C, declustering potential 71 V, collision energy 21 V. Raw data analysis was performed using Analyst v1.3.1 (Applied Biosystems, Foster City, CA).

LC/MS with Accurate Mass Measurement. Microsomal samples for analysis were evaporated to dryness and reconstituted in acetonitrile/0.1% formic acid (50:50). Sample separation was achieved using a Hewlett-Packard 1100 HPLC system with gradient capabilities. A Varian Polaris column (C18-A 3-μm, 2.1 × 150 mm; Varian, Inc., Palo Alto, CA) was used with a sample injection volume of 50 μl. The mobile phase system consisted of 5 mM ammonium formate (pH 3.0) in water with 1% acetonitrile (solvent A) and acetonitrile (solvent B). The gradient conditions were 5% B, initial for 5 min, 5 to 100% B in 16 min, 100% B for 1.0 min, 100% to 5% B in 0.1 min, then hold at 5% B for 2.9 min. Samples were analyzed using a Micromass quadrupole time of flight (Q-ToF2) instrument equipped with lockspray in positive ion electrospray mode. The instrument was operated in V-mode with approximately 10,000 resolution. Metabolites (Sigma-Aldrich) was used as a lockmass compound for mass spectrometry and tandem mass spectrometry accurate mass measurements. The instrument parameters were set as follows: cone 50 V, capillary 3.25 kV, collision 10 V (27.5 for tandem mass spectrometry), source temperature 100°C, desolvation temperature 250°C. Raw data analysis was performed using MassLynx 3.5 (Waters).

Cleland’s Reagent (Dithiothreitol, DTT). DTT (100 mM) was added to an incubated and cleaned up HLM sample (100 μl) containing pioglitazone and excess GSH (100 μl; final concentration of DTT was 50 mM) and set at room temperature overnight. Sample analysis and separation were accomplished as stated above for accurate mass measurements. A HLM sample containing pioglitazone and excess GSH without DTT set at room temperature overnight was used as a control.

Results

A summary of the novel metabolites, M-A and M-B, detected from incubations with rat and human liver microsomes and freshly isolated rat hepatocytes, is shown in Table 1. No glutathione conjugates were detected in freshly isolated (n = 1) or cryopreserved (n = 2) human hepatocytes. To characterize the metabolism of pioglitazone, we used...
two in vitro systems (microsomes and hepatocytes) and two analysis methods (LC/MS with neutral loss scanning and LC/MS Q-ToF accurate mass measurement). Glutathione conjugates, M-A and M-B, were first detected using LC/MS with neutral loss scanning and then further identified by LC/MS Q-ToF accurate mass measurements.

A review of the literature showed that pioglitazone is metabolized to form several oxidative products and a cleavage product at the ether linkage (Krieter et al., 1994; Sohda et al., 1995; Tanis et al., 1996; Kiyota et al., 1997; Eckland, 2000; Shen et al., 2003). A majority of the oxidative metabolites previously reported for pioglitazone and conjugates M-A and M-B were detected in rat and human liver microsomes and hepatocytes in our laboratory (Figs. 2 and 3). Shen et al. (2003) identified a thiazolidinedione ring-opened product (M-X) of pioglitazone in dog liver microsomes. This study reports the thiazolidinedione ring-opened product (M-X) identified in rat and human liver microsomes and two (M-A and M-B) novel thiazolidinedione ring-opened glutathione conjugates of pioglitazone detected in rat and human liver microsomes and freshly isolated rat hepatocytes.

**Microsomal Studies.** Both rat and human liver microsomes resulted in two peaks in the analysis corresponding to the typical 129-amu neutral loss of glutathione conjugates, which led to further identification by LC/MS Q-ToF accurate mass measurements. Further analysis using rat and human liver microsomal incubation (5 ml) with pioglitazone, NADPH, and GSH was performed to increase sensitivity for detection of accurate mass for M-A, M-B, and M-X.

The extracted ion chromatogram of M-A and the product ion spectrum are shown in Fig. 4, A and B, respectively. M-A presented a [M + H]+ ion of 636.2139 by LC/MS accurate mass measurement, which corresponded to a molecular formula of C28H38N5O8S2 (3.5 ppm). The product ion spectrum provided a typical neutral loss fragmentation of glutathione conjugates, m/z 561 (−75 amu) and m/z 507 (−129 amu). The accurate mass fragmentation outlined in Fig. 5 supports the proposed structure of M-A.

The extracted ion chromatogram of M-B and the product ion spectrum are shown in Fig. 6, A and B, respectively. M-B presented
a [M + H]^+ ion of m/z 637.1979 by LC/MS accurate mass measurement which corresponded to a molecular formula of C_{28}H_{37}N_{4}O_{9}S_{2} (3.5 ppm). The product ion spectrum provided a typical neutral loss fragmentation of glutathione conjugates, m/z 562 (-75 amu) and m/z 508 (-129 amu). The accurate mass fragmentation outlined in Fig. 7 supports the proposed structure of M-B.

The extracted ion chromatogram of M-X and the product ion spectrum are shown in Fig. 8, A and B, respectively. M-X presented a [M + H]^+ ion of m/z 379 by LC/MS accurate mass measurement. The product ion spectrum gives a typical pattern showing a thiazolidinedione ring-opened product, m/z 299.1757 [M + H - SO_3]$, which corresponded to a molecular formula of C_{18}H_{23}N_{2}O_{2} (0.9 ppm), and m/z 282.1448 [M + H - SO_3 - NH_3]^+, which corresponded to a molecular formula of C_{18}H_{20}NO_{2} (16.4 ppm). The product ion m/z 134.0912 suggests that the pyridyl ethyl end of the molecule was unconjugated. This is the first detection of this metabolite of pioglitazone in rat and human in vitro systems.

Further positive identification of M-A and M-B was performed using Cleland’s reagent, DTT, which is known to reduce disulfide bonds (Cleland, 1964). The effect of using DTT is shown in Fig. 9, where the chromatographic peaks for M-A and M-B are present in the control (Fig. 9A) and are approximately 96% reduced in the extracted ion chromatogram from an incubation of pioglitazone in HLMs with excess GSH and DTT (Fig. 9B).

**Hepatocyte Studies.** Hepatocyte extracts were also analyzed by LC/MS with neutral loss scanning, multiple reaction monitoring (MRM), and accurate mass measurements. M-A and M-B were detected in freshly isolated rat hepatocytes by neutral loss scanning and confirmed by MRM, comparison to control, and retention times of M-A and M-B isolated from rat and human liver microsomes. Glutathione conjugates, M-A and M-B, were not detected in freshly isolated or cryopreserved human hepatocytes; however, several of the known oxidative metabolites of pioglitazone were detected (Fig. 2). The metabolites detected were M-IX, the -2H metabolite; M-III, the keto metabolite; M-II, M-IV, M-VII, and M-VIII, the hydroxy metabolites; and M-V, the carboxylic acid metabolite on the terminal carbon of the ethyl side chain. Metabolites labeled in Figs. 2 and 3 were labeled using the identifiers from Shen et al. (2003).
Discussion

Three thiazolidinedione ring-opened metabolites of pioglitazone have been identified by mass spectrometry in rat and human liver microsomes and in freshly isolated rat hepatocytes, two of which are glutathione conjugates. The ring-opened products were not positively identified in freshly isolated human hepatocytes or cryopreserved human hepatocytes, even though the cells were not deficient in GSH activity as determined by a positive control, troglitazone. In each hepatocyte study, troglitazone was used as a positive control monitoring the MRM transition 747.0/440.0 (loss of GSH). This metabolite of troglitazone has previously been identified in cryopreserved human hepatocytes by Prabhu et al. (2002). The glutathione conjugate of troglitazone was identified in each hepatocyte study (data not shown). This finding suggests that the in vitro glutathione conjugates of pioglitazone are specific to rat liver microsomes and hepatocytes, dog liver microsomes (Shen et al., 2003), and human liver microsomes. It is possible that these ring-opened products of pioglitazone are formed in vivo in human liver but are not of significant consequence, either because they are not relevant to toxicity or because there are not significant amounts of the reactive species to cause toxicity.

The identified metabolically activated products of pioglitazone may have some clinical relevance for possible causes of liver injury; however, more extensive work would have to be performed to determine this. Therefore, the toxicological relevance of this finding is unclear, mainly because pioglitazone is a much safer drug, from a human liver toxicity perspective, compared with troglitazone. The safety profile of pioglitazone is much improved over troglitazone, which might be a result of dosage, since pioglitazone is administered at approximately 10 times lower dosage compared with troglitazone.

This is the first report of a metabolically activated thiazolidinedione ring-opened product of pioglitazone in rat and human in vitro systems. The proposed structures of metabolites detected in this study are reported in Fig. 10. Metabolites M-A, M-B, and M-X go through an oxidation step and then loss of carbon dioxide. Then, the remaining amide-sulfenic acid intermediate can be further oxidized to M-X or can go through nucleophilic substitution by GSH and lose a molecule of water to get M-A as a product.

Evidence exists in the literature that thiazolidinedione rings are known to be reactive species upon S-oxidation (Kassahun et al., 2001;...
Nelson, 2001), and pioglitazone follows in line in generating a ring-opened reactive species in vitro. As discussed earlier, troglitazone has been found to be hepatotoxic and removed from the market. There have been rare reports of clinically significant hepatocellular dysfunction and cholestatic liver injury in patients undergoing pioglitazone treatment (Maeda, 2001; Chase and Yarze, 2002; May et al., 2002). It is possible that these metabolites may contribute to these very rare reports of liver injury in an idiosyncratic fashion.

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References


