Mouse Red Blood Cell-Mediated Rare Xenobiotic Phosphorylation of a Drug Molecule Not Intended to Be a Kinase Substrate[§]

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ABSTRACT

Phosphorylation of xenobiotics is rare, probably owing to a strong evolutionary pressure against it. This rarity may have attracted more attention recently as a result of intentionally designed kinase-substrate analogs that depend on kinase-catalyzed activation to form phosphorylated active drugs. We report a rare phosphorylated metabolite observed unexpectedly in mouse plasma samples after an oral dose of a Tankyrase inhibitor that was not intended to be a kinase substrate, i.e., (S)-2-(4-(6-(3,4-dimethylpiperazin-1-yl)-4-methylpyridin-3-yl)phenyl)-8-(hydroxymethyl)quinazolin-4(3H)-one (AZ2381). The phosphorylated metabolite was not generated in mouse hepatocytes. In vitro experiments showed that the phosphorylation of AZ2381 occurred in mouse whole blood with heparin as anticoagulant but

not in mouse plasma. The phosphorylated metabolite was also produced in rat, dog, and human blood, albeit at lower yields than in mouse. Divalent metal ions are required for the phosphorylation since the reaction is inhibited by the metal chelator EDTA. Further investigations with different cellular fractions of mouse blood revealed that the phosphorylation of AZ2381 was mediated by erythrocytes but did not occur with leukocytes. The levels of ¹⁸O incorporation into the phosphorylated metabolite when inorganic ¹⁸O₄-phosphate and γ -¹⁸O₄-ATP were added to the mouse blood incubations separately suggested that the phosphoryl transfer was from inorganic phosphate rather than ATP. It remains unclear which enzyme present in red blood cells is responsible for this rare phosphorylation.

Introduction

Phosphorylation of xenobiotics is rare, probably owing to a strong evolutionary pressure against it (Parkinson et al., 2013). This has been rationalized for a number of reasons (Parkinson, 2001; Parkinson et al., 2013; Mitchell, 2016), such as limiting consumption of ATP in cells by xenobiotics, avoiding interference with intracellular signaling, and assuming that the phosphorylated metabolites are probably not excreted through the cell membrane if formed in hepatocytes or other cells. The topic of xenobiotic phosphorylation has attracted more attention recently owing to intentionally designed kinase-substrate analogs that depend on kinase-catalyzed activation to form phosphorylated active drugs. Examples include a number of antiviral and anticancer drugs that are nucleoside mimetics requiring phosphorylation to activate, as well as the immunomodulatory drug fingolimod (Kalász et al., 2013; Mitchell, 2016). Fingolimod is a structural analog of sphingosine and is phosphorylated by sphingosine kinases to become an agonist of the sphingosine-1-phosphate receptor (Mandala et al., 2002; Billich et al., 2003; Zollinger et al., 2011; David et al., 2012). However, the phosphorylation of fingolimod may still be viewed as an endogenous pathway for the closely related structural analog of a kinase substrate,

rather than as a biotransformation typical for xenobiotics (Zollinger et al., 2011). Recently, Scheible et al. (2017) have reported a novel conjugation metabolite with phosphoethanolamine as a major metabolite of the mitogen-activated protein kinase kinase (MEK)1/2 inhibitor pimasertib in plasma and feces of cancer patients. They have suggested that the novel conjugation with phosphoethanolamine may result from the structural similarity to glycerol of the propanediol moiety of pimasertib, which thus leads to lipid metabolism (Scheible et al., 2017). Previously, Zollinger et al. (2008) have described a phosphocholine conjugate of the immunosuppressant everolimus which was the most prominent drug metabolite in human and preclinical animal blood. The phosphocholine conjugation following the hydroxylation of a bicyclo[1.1.1]pentane of hepatitis C NS5B inhibitors has also been observed in rat bile by Zhuo et al. (2016). Both cases of phosphocholine conjugation involve phospholipid synthesis metabolism of xenobiotics that do not appear to have close structural similarity to the endogenous substrates (Zollinger et al., 2008; Zhuo et al., 2016).

A pyrimidinone nicotinamide mimetic compound (S)-2-(4-(6-(3,4-dimethylpiperazin-1-yl)-4-methylpyridin-3-yl)phenyl)-8-(hydroxymethyl)quinazolin-4(3H)-one (AZ2381) was identified as an oral lead compound that selectively inhibits the poly-ADP-ribose polymerase catalytic domain of Tankyrases and thus potentially inhibits the Wnt pathway (Johannes et al., 2015). In other words, AZ2381 was not designed to be a kinase substrate. When the intended administration route was altered from oral to intravenous during drug discovery (Johannes et al., 2015), a phosphate

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ABBREVIATIONS: AZ2381, (S)-2-(4-(6-(3,4-dimethylpiperazin-1-yl)-4-methylpyridin-3-yl)phenyl)-8-(hydroxymethyl)quinazolin-4(3H)-one; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; LC, liquid chromatography; *m*-CPBA, *m*-chloroperoxybenzoic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PD, pharmacodynamics; PK, pharmacokinetics; RBC, red blood cells (also called erythrocytes); SCID, severe combined immunodeficiency; THF, tetrahydrofuran.

Scheme 1. AZ2381 and phosphorylated AZ2381.

prodrug of AZ2381 at the benzyl alcohol (Scheme 1) was made to test intravenous administration, as the poor solubility of AZ2381 meant that it is not suitable for intravenous administration. During the studies comparing the intravenously dosed prodrug with orally dosed AZ2381 in mouse, phosphorylated AZ2381 was unexpectedly detected in mouse plasma samples collected from the AZ2381 dose groups. The present study has confirmed that, indeed, a rare xenobiotic phosphorylation metabolite was formed in mouse. Perhaps even more surprising, the phosphorylation is found to be mediated by red blood cells, and the source of the phosphoryl group appears to be inorganic phosphate.

Materials and Methods

AZ2381 was synthesized as described by Johannes et al. (2015), compound **15** in the Supporting Information of the reference). Chemical synthesis of phosphorylated AZ2381 is described here (see Scheme 2 for the two-step synthetic route).

(S)-Di-tert-butyl(2-(4-(6-(3,4-dimethylpiperazin-1-yl)-4-methylpyridin-3-yl)phenyl)-4-oxo-3,4-dihydroquinazolin-8-yl)methyl phosphate (di-tert-butyl-phosphorylated AZ2381). 1H-Tetrazole (0.45 M in acetonitrile, 8 ml) and di-tert-butyl diethylphosphoramidite [591 mg in 5 ml tetrahydrofuran (THF)] were added to a solution of AZ2381 (180 mg) in 10 ml of THF. The resulting mixture was stirred at room temperature for 50 hours. Liquid chromatography-mass spectrometry (LC-MS) indicated the completion of reaction. The reaction mixture was cooled to -78°C , and *m*-chloroperoxybenzoic acid (*m*-CPBA; 100 mg, 77% in THF, 5 ml) was added to the mixture dropwise. The mixture was stirred at -78°C for 10 minutes and then the cooling bath was removed. The reaction mixture was checked by LC-UV-MS, which indicated the formation of product and about 22% of intermediate. The mixture was cooled back to -78°C , and 40 mg of *m*-CPBA in 1 ml of THF was added to the mixture, which was then stirred for 5 minutes. LC-MS indicated completion of the oxidation. To the resulting mixture was added a solution of saturated NaHCO_3 , followed by dilution with ethyl acetate. The layers were separated, the organic layer was dried with anhydrous Na_2SO_4 , concentrated, and the residue was purified via normal phase chromatography on silica gel (eluted with 100% dichloromethane to 20% methanol in dichloromethane) to yield the desired product, (S)-di-tert-butyl(2-(4-(6-(3,4-dimethylpiperazin-1-yl)-4-methylpyridin-3-yl)phenyl)-4-oxo-3,4-dihydroquinazolin-8-yl)methyl phosphate (250 mg, 98%). MS: $[\text{M}+\text{H}]^+$ m/z 648.6. ^1H NMR (400 MHz, methanol- d_4): δ ppm 8.67 (s, 1 H) 8.19–8.32 (m, 3 H) 8.03 (s, 1 H) 7.97 (dd, $J = 7.28$, 1.00 Hz, 1 H) 7.47–7.63 (m, 3 H) 6.89 (s, 1 H) 5.63 (d, $J = 7.28$ Hz, 2 H) 4.33–4.36 (m, 2 H) 3.23–3.43 (m, 3 H) 3.02–3.06 (m, 2 H) 2.81 (s, 3 H) 2.34 (s, 3 H) 1.50 (s, 18 H) 1.40–1.41 (d, 3 H).

(S)-2-(4-(6-(3,4-Dimethylpiperazin-1-yl)-4-methylpyridin-3-yl)phenyl)-4-oxo-3,4-dihydroquinazolin-8-yl)methyl dihydrogen phosphate (phosphorylated AZ2381). The di-tert-butyl-phosphorylated AZ2381 (250 mg, 0.39 mmol) was dissolved into 4 ml of dichloromethane, followed by addition of 4 ml of 4 M HCl in 1,4-dioxane. The mixture was stirred for 10 minutes. The mixture was concentrated, and the residue was purified by reverse-phase preparative LC (a C18 column eluted with 0–30% of 0.1% formic acid in acetonitrile/0.1% formic acid in water) to yield phosphorylated AZ2381 (100 mg, 45.3%). High resolution MS, $[\text{M}+\text{H}]^+$ m/z 536.2071 (calculated exact mass 536.2057, mass error 2.6 ppm). ^1H NMR (400 MHz, DMSO- d_6): δ ppm 1.21 (d, $J = 5.52$ Hz, 3 H) 2.19 (s, 3 H) 2.55 (s, 3 H) 2.67 (br s, 2 H) 3.00 (br s, 1 H) 3.15 (br s, 2 H) 4.28 (br s, 2 H) 5.39 (d, $J = 6.27$ Hz, 2 H) 6.84 (s, 1 H) 7.32–7.55 (m, 3 H) 7.88 (d, $J = 7.53$ Hz, 1 H) 7.95 (s, 1 H) 8.04 (d, $J = 7.78$ Hz, 1 H) 8.25 (d, $J = 8.28$ Hz, 2 H). ^{31}P NMR (300 MHz, DMSO- d_6): δ ppm 0.03.

Chemicals, Biochemicals and Media. Ammonium-chloride-potassium (ACK) lysing buffer (Gibco cat. no. A1049201) was bought from Thermo Fisher Scientific (Waltham, MA). ATP disodium salt hydrate (product no. A1852), Dulbecco's phosphate-buffered saline (DPBS; without Ca^{2+} and Mg^{2+} , product no. D8537), and RPMI-1640 cell culture medium (product no. R8758) were purchased from Sigma-Aldrich (St. Louis, MO). EDTA, pH 7.4, solution (0.5 M, prepared in 18.2 MΩ water, pH adjusted with sodium hydroxide) were bought from Boston BioProducts (Ashland, MA). Ficoll-Paque Plus centrifugation medium was made by GE Healthcare (Chicago, IL). γ - $^{18}\text{O}_4$ -ATP sodium salt (cat. no. OLM-7858, lot no. PR-22511), $^{18}\text{O}_4$ -phosphoric acid in ^{18}O -water (cat. no. OLM-1057), and ^{18}O -water (cat. no. OLM-240-97) were obtained from Cambridge Isotope Laboratories (Andover, MA). An $^{18}\text{O}_4$ -phosphate stock buffer, pH 7.4, was prepared in unlabeled water by titrating 50 mM $^{18}\text{O}_4$ -phosphoric acid with a 100 mM potassium hydroxide using a pH meter equipped with a Micro pH Electrode.

Blood, Plasma, and Cellular Fractions. Mouse blank blood [CB17 severe combined immunodeficiency (SCID) or Nude] was collected at our laboratory into BD Microtainer PST tubes with lithium heparin (Becton, Dickinson and Company, Franklin Lakes, NJ). The heparinized mouse plasma was separated from the heparinized whole blood by centrifugation. Rat blood (Nude) was also freshly collected at our laboratory into the same heparin-coated tube.

Total leukocytes in the mouse blood were collected by lysing red blood cells (RBC, also called erythrocytes) using the following procedures at room temperature unless specified: Pipetted 1 ml heparin-treated whole blood into a tube containing 10–20 ml of ACK lysing buffer. Allowed the mixture to incubate for 3–5 minutes. Collected the leukocytes by centrifugation at 300g for 5 minutes. Aspirated the supernatant, leaving approximately 50 μl to avoid disturbing the pellet. Gently mixed the cells and the remaining fluid, then added 5 ml cold DPBS. Mixed the cells and DPBS, and then collected the cells by centrifugation at 300g

Scheme 2. Chemical synthesis of phosphorylated AZ2381.

for 5 minutes at 2–8°C. Aspirated the supernatant and resuspended the leukocytes in RPMI-1640 cell culture medium.

Mouse leukocytes and RBCs were also separated from the whole blood using a “buffy coat” protocol (<http://www.humanimmunologyportal.com/protocols/preparing-a-buffy-coat-from-whole-blood/>)—added 1 part DPBS in 1 part fresh whole blood and mixed well, centrifuged the diluted blood at 200g at room temperature for 10 minutes with the brake off, collected the leukocyte thin band (i.e., buffy coat) along with a small portion of the plasma above it and a small portion of RBCs beneath it, and collected the remaining RBCs into another tube. The buffy coat band collection was further separated using a Ficoll-Paque protocol (GE Healthcare https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/ge-isolation-of-mononuclear-cells.pdf) to remove the residual RBCs. Mononuclear leukocytes above Ficoll-Paque medium and remaining polymorphonuclear leukocytes in Ficoll-Paque medium were combined after a further centrifugation, which resulted in a combined leukocyte fraction that was free of RBC contamination, although some of polymorphonuclear leukocytes may have been lost in the RBC band beneath the Ficoll-Paque media. All isolated cellular fractions were resuspended in RPMI-1640 cell culture medium to the equal volume of the original blood sample.

Mouse In Vivo Study #1: A Toxicology Study in CD-1 Mice. AZ2381 was formulated in 15% hydroxypropyl- β -cyclodextrin in water-for-injection adjusted to pH 2.0. It was orally administrated to CD-1 female mice at 25 and 90 mg/kg, respectively, with a dosing volume of 10 ml/kg. Blood samples of 20 μ l were harvested at 0.5, 1, and 4 hours after respective doses, via tail vein puncture using 20 μ l end-to-end glass capillaries precoated with K2-EDTA (order no. 19.447; Sarstedt, Nümbrecht, Germany) and then transferred to Eppendorf tubes preloaded with 80 μ l of PBS. The PBS-diluted blood samples were centrifuged at 14,500g at 4°C for 5 minutes to separate the diluted plasma. The plasma samples of 75 μ l were then transferred to storage tubes and frozen at –20°C before analysis. After the bioanalysis, residual plasma samples collected from four mice at 1 hour after the 90-mg/kg dose were pooled at equal volumes. The pooled sample was combined with two volumes of acetonitrile. After centrifugation, the resulting clear supernatant was transferred to an autosampler vial and an aliquot of 10 μ l was injected for LC-UV-MS drug metabolite analysis.

Mouse In Vivo Study #2: A Pharmacokinetic Study in SCID Mice. AZ2381 was orally administrated to CB17 SCID female mice at 90 mg/kg using the same formulation and dosing volume as in the in vivo study #1. Blood samples were collected by submandibular bleeding at multiple time points to determine pharmacokinetic (PK) parameters. K2-EDTA was used as anticoagulant. Plasma samples separated via centrifugation of the blood were stored at –20°C before analysis. After the bioanalysis, residual mouse plasma samples collected from three animals at 1 hour were pooled at equal volumes. The pooled sample was processed as described in the in vivo study #1 for drug metabolite analysis.

Mouse In Vivo Study #3: A Pharmacodynamics Study in SCID Mice. AZ2381 was dosed orally to CB17 SCID female mice at 25 and 90 mg/kg using the same formulation and dosing volume as in the in vivo studies #1 and #2. Blood samples of 20 μ l were harvested at 5 minutes, and 0.5, 1, 2, 8, and 24 hours after respective doses, via tail vein puncture using 20- μ l end-to-end glass capillaries precoated with K2-EDTA (Sarstedt) and then transferred to microplate glass insert tubes on ice. Total animals included in the 25- and 90-mg/kg dose groups were four and six, respectively. However, to minimize the volume of blood withdrawn from individual mice in the pharmacodynamics (PD) study, only two and three animals of the respective 25- and 90-mg/kg group were sampled for blood at each time point. After centrifugation of blood samples at 14,500g at 4°C for 5 minutes, separated plasma samples of 5 μ l were transferred to storage tubes, followed by the addition of 20 μ l of commercially available blank CD-1 mouse plasma, and then frozen at –20°C before bioanalysis.

In Vitro Incubations of AZ2381 in Mouse Blood and Plasma. AZ2381 of 10 μ M concentration was incubated in mouse whole blood with heparin as the anticoagulant, in the mouse blood fortified with 5 mM ATP and 10 mM MgCl₂, in the mouse blood in the presence of 100 mM EDTA, and in the heparinized mouse plasma fortified with 5 mM ATP and 10 mM MgCl₂, respectively, in capped vials at 37°C in a shaking water bath. Individual incubation volumes were 250 μ l. The incubations were stopped at 2 hours by adding equal volumes of acetonitrile. Clear supernatant after centrifugation was transferred to a 96-well plate and diluted with an equal volume of water. Aliquots of 10 μ l were injected for LC-UV-MS analysis.

Additionally, a negative control experiment was conducted in DPBS with 5 mM ATP and 10 mM MgCl₂ added. In all in vitro incubations described in this article, AZ2381 was spiked from a 10 mM stock solution in dimethyl sulfoxide (DMSO) (i.e., only 0.1% DMSO was introduced to the incubations), ATP and MgCl₂ stock solutions were freshly prepared in DPBS, and EDTA was added from a pH 7.4 buffer solution. When applicable, blank DPBS was added to make up the total volume so that the same slight dilution of blood sample would be applied for all incubation conditions in one in vitro experiment.

In Vitro Incubations of AZ2381 in Mouse Leukocytes and RBCs. AZ2381 of 10 μ M concentration was incubated in mouse whole blood, and in the total cellular fraction after removal of plasma, in leukocytes and in RBCs, respectively. The mouse blood and whole cellular fractions were fortified with 5 mM ATP and 10 mM Mg²⁺ for the incubation. The leukocyte and RBC incubations were carried out with or without adding 10 mM ATP and 10 mM MgCl₂. Additionally, AZ2381 was incubated in ATP- and Mg²⁺-fortified RBCs in the presence of 100 mM EDTA. All incubations were conducted in triplicate with individual volumes at 250 μ l in a covered 96-well plate at 37°C in a shaking water bath. A negative control experiment was conducted in the blank medium supplemented with 10 mM ATP and 10 mM MgCl₂. The incubations were stopped at 2 hours by adding equal volumes of acetonitrile. A 400- μ l portion of clear supernatant after centrifugation was transferred to a 96-well plate and diluted with an equal volume of water. Aliquots of 15 μ l were injected for LC-UV-MS analysis.

Mouse Blood Incubations of AZ2381 in the Presence of γ -¹⁸O₄-ATP, Inorganic ¹⁸O₄-Phosphate, or ¹⁸O-Water. AZ2381 of 10 μ M was incubated at 37°C in the mouse blood supplemented with γ -¹⁸O₄-ATP in DPBS (final concentration, 10 mM), with inorganic ¹⁸O₄-phosphate buffer, pH 7.4, in unlabeled water (final concentration, approximately 6 mM), or with ¹⁸O-water (final concentration, 10 M). In each of the incubations, mouse blood of 200 μ l was supplemented with the 50- μ l volume of γ -¹⁸O₄-ATP stock solution in DPBS, inorganic ¹⁸O₄-phosphate buffer, or ¹⁸O-water. The reactions were terminated after 2 hours and the samples were processed as described in the experiment above. Additionally, a control experiment to test chemical exchange of oxygen was conducted by incubating 2 μ M synthetic standard of phosphorylated AZ2381 in duplicate with approximately 6 mM ¹⁸O₄-phosphate and 10 M ¹⁸O-water in a DPBS solution at 37°C for 2 hours.

LC-MS/MS Quantitative Bioanalysis of Plasma Samples of Mouse In Vivo Studies. Calibration standards were prepared with an appropriate concentration range in blank CD-1 mouse plasma (K3-EDTA; Bioreclamation-IVT, Westbury, NY). Three quality control samples at low, medium, and high concentrations were also included in the bioanalysis. Mouse plasma samples were analyzed using a protein precipitation extraction procedure with acetonitrile containing an internal standard compound, followed by LC-MS/MS quantification using a C18 column with a Shimadzu LC-20AD LC system (Shimadzu Scientific Instruments, Columbia, MD) and an API 4000 triple quadrupole mass spectrometer and processed with Analyst (AB Sciex, Framingham, MA). Selected reaction monitoring in positive-ion mode was used for the detection of AZ2381, phosphorylated AZ2381, and internal standard in the LC-MS/MS. More specifically, AZ2381 and phosphorylated AZ2381 were monitored by ion transitions of m/z 456.2 $\rightarrow$ 354.2 and m/z 536.2 $\rightarrow$ 438.2, respectively.

LC-UV-MS and MS/MS Metabolite Profiling and Identification. The metabolite analysis was conducted using an LC-UV-MS system consisting of an Acquity UPLC system (Waters, Milford, MA) and an LTQ-Orbitrap XL high-resolution mass spectrometer equipped with an electrospray ionization source (Thermo Fisher Scientific). An LC column of Aquasil C18, 3 μ M, 100 $\times$ 2.1 mm (Thermo Fisher Scientific) was used. The mobile phase at a flow rate of 0.250 ml/min consisted of water (A) and acetonitrile (B), both containing 0.1% (v/v) formic acid. The LC gradient started at 5% B and was maintained for 2 minutes, then increased linearly to 30% during the next 18 minutes. It was followed by a linear increase to 98% B over the next 3 minutes and kept at 98% B for 2 minutes, finally decreased to 5% B and equilibrated for 7 minutes before next injection. The UV wavelength at 315 nm with 6-nm resolution was monitored in acquisition of UV chromatograms, and photodiode array UV spectra of 200–500 nm were also acquired. LC-MS spectra were acquired at the resolution of 15,000. Collision-induced dissociation (CID) with helium as collision gas in the linear ion trap was used in acquisition of MS/MS spectra. The normalized collision energy at 20 of the manufacturer's unit and the activation time of 30 milliseconds were used in CID experiment.

Fig. 1. Mouse plasma metabolite profile of AZ2381 at 1 hour after an oral dose of 90 mg/kg, recorded in UV chromatogram at 315 nm (A), and extracted ion chromatogram with the peaks marked with accurate mass of protonate molecular ions, i.e., m/z of $[M+H]^+$ ions (B).

Results

Several metabolites of AZ2381 were observed in the pooled mouse plasma sample collected at 1 hour after an oral dose of 90 mg/kg to CD-1 mice in a toxicology study (Fig. 1), including common oxidation metabolites undergoing *N*-demethylation (M1), a further *N*-hydroxylation following the *N*-demethylation (M3), *N*-oxidation (M2), and glucuronidation of the parent drug, M1 and M2 (m/z 632, 618, and 648, respectively, Fig. 1). In addition, an unexpected phosphorylation metabolite was detected in the mouse plasma (phosphorylated AZ2381, Fig. 1). This phosphorylated metabolite was proven to be identical to the chemically synthesized standard by LC-UV-MS and MS/MS (Supplemental Fig. 1: the same retention time, the same accurate mass of $[M+H]^+$, and the same MS/MS fragmentation pattern). In addition, a plasma metabolite profile similar to AZ2381 including the same rare phosphorylated metabolite was observed in a SCID mouse pooled plasma sample collected at 1 hour after a 90-mg/kg oral dose in a PK study (data not shown).

Figure 2 shows the plasma PK profile of AZ2381 up to 24 hours after an oral dose of 25 and 90 mg/kg, respectively, to SCID mice in a single-dose PD study (data points and lines in blue). Also plotted are phosphorylated AZ2381 detected in the mouse plasma of both dose groups. The plasma concentration of phosphorylated AZ2381 was time-dependent and dose-dependent (data points and lines in red, Fig. 2). The area under the curve of phosphorylated metabolite was determined to be approximately 5–10% that of the parent drug in respective dose groups. Additionally, the dosing formulation check performed during the bioanalysis indicated no trace of contamination by the phosphate prodrug investigated in the same PD study (Supplemental Fig. 2).

The phosphorylated metabolite was generated by the incubation of AZ2381 in mouse whole blood with heparin as the anticoagulant (Fig. 3A). Addition of 5 mM ATP and 10 mM Mg^{2+} to the whole blood did not cause a noticeable change in the yield of phosphorylated metabolite (Fig. 3B). However, the presence of divalent metal ion chelator EDTA diminished the phosphorylation by 84% (Fig. 3C vs. Fig. 3A). Mouse

plasma did not produce any phosphorylated metabolite, in contrast to the whole blood under the same incubation conditions (Fig. 3D vs. Fig. 3B). The phosphorylated metabolite was not detected after a control incubation in a DPBS solution supplemented with ATP and Mg^{2+} . In addition, the phosphorylated metabolite was not produced in mouse hepatocytes, when oxidation metabolites (e.g., M1, M2, and M3) and glucuronidation metabolites occurred after the incubation of 10 μ M AZ2381 in 2×10^6 cells/ml CD-1 mouse cryopreserved hepatocytes at 37°C for 2 hours (data not shown). The extracted ion chromatograms in Fig. 3 were constructed by accurate masses of all ions generated in the

Fig. 2. Plasma mean concentration of AZ2381 parent drug (blue) and phosphorylated AZ2381 metabolite (red) as the function of time after an oral dose of 25 mg/kg AZ2381 (solid dots) or 90 mg/kg AZ2381 (open squares) in mice. Error bars of the 90-mg/kg dose group are plotted as the S.D. of triplicate animal samplings. No error bars are provided for the 25-mg/kg dose group as only duplicate animal samplings were made.

Fig. 3. LC-MS accurate-mass-extracted ion chromatograms following a 2-hour incubation of 10 μ M AZ2381 at 37°C in mouse blood with heparin as anticoagulant (A), the mouse blood fortified with 5 mM ATP and 10 mM MgCl₂ (B), the mouse blood with 100 mM EDTA added (C), and the heparinized mouse plasma fortified with 5 mM ATP and 10 mM MgCl₂ (D), and leukocytes collected after lysing red blood cells in the mouse blood and fortified with 5 mM ATP and 10 mM MgCl₂ (E). Displayed are extracted ion chromatograms consisting of singly and doubly protonated molecular ions of the parent drug and the metabolite, as well as an in-source fragment ion of doubly protonated metabolite via the neutral loss of a H₃PO₄.

electrospray ionization source for the parent drug and phosphorylated metabolite, including singly and doubly protonated molecular ions (m/z 456.239 and 228.623 of the parent, m/z 536.206 and 268.607 of the phosphorylated metabolite), as well as an in-source fragment ion (m/z 219.618, formed by the neutral loss of an H₃PO₄ from the doubly protonated metabolite). The accurate-mass extracted ion chromatogram in Fig. 3 provides a highly selective display for the occurrence or the absence of the phosphorylation in the incubations, with superb signal-to-noise when chemical noise was eliminated by use of high-resolution filtering.

The total leukocytes collected after lysing RBCs in the mouse blood using an ACK lysing buffer did not generate the phosphorylated metabolite (Fig. 3E). This negative result prompted us to isolate intact RBCs to demonstrate with positive results that the phosphorylation was indeed mediated by mouse RBCs. Bar chart Fig. 4 provides quantitative comparisons for the formation of phosphorylated AZ2381 in mouse blood and different cellular fractions, after the incubation of 10 μ M AZ2381 at 37°C for 2 hours. The cellular fractions were resuspended in

a cell culture medium to the original volume of whole blood from which each cellular fraction was isolated. The percentages in the figure were calculated from LC-UV peak areas with an adjustment by different UV response of phosphorylated-AZ2381 versus parent drug AZ2381 (at an absorbance ratio of approximately 1.2 at 315 nm). A negative control incubation conducted in the blank medium supplemented with ATP and MgCl₂ did not show any occurrence of the phosphorylation. The removal of plasma from the whole blood increased the yield of phosphorylation (“Without Plasma” vs. “Whole Blood” in Fig. 4). Leukocytes did not produce any phosphorylated AZ2381 (the zero bar length for leukocytes in Fig. 4; also not detected in LC-MS accurate-mass extracted ion chromatograms that are not shown). According to similar phosphorylation yields in three RBC incubations shown in Fig. 5, additional ATP or Mg²⁺ introduced to the RBC incubation did not affect the phosphorylation yield; however, the presence of EDTA diminished the phosphorylation (Fig. 4). This is similar to what was observed with whole blood incubates as shown in Fig. 3. To identify the enzyme in RBC responsible for the phosphorylation, the identification

Fig. 4. Percent yields of phosphorylated AZ2381 determined by LC-UV signal responses of the metabolite and remaining parent drug after an incubation of 10 μ M AZ2381 at 37°C for 2 hours in mouse whole blood with heparin as anticoagulant, the total cellular fractions after removing plasma, leukocytes, and red blood cells (RBC). The bar chart is plotted as the mean $\pm$ S.D. of triplicate incubations. These incubations were fortified with 5–10 mM ATP and/or 10 mM MgCl₂.

of the source of phosphoryl transfer would have been helpful. Stable isotope ^{18}O -labeled ATP and inorganic phosphate were used for the identification, together with ^{18}O -labeled water to investigate if chemical exchange of ^{18}O would occur. Figure 5 compares the LC–high resolution MS spectra of the protonated molecular ion region recorded for the phosphorylated metabolite produced by the incubation of AZ2381 in mouse blood (panel A) versus the mouse blood with γ - $^{18}\text{O}_4$ -ATP (10 mM, panel B) added, or inorganic $^{18}\text{O}_4$ -phosphate buffer pH 7.4 (approximately 6 mM, panel C), or ^{18}O -water of high concentration (10 M, panel D). The inorganic $^{18}\text{O}_4$ -phosphate pH 7.4 buffer was prepared in unlabeled water. When γ - ^{18}O -labeled ATP material was added to the mouse blood incubation, additional phosphorylated metabolite ions were observed corresponding to the $[\text{M}+\text{H}]^+$ ions of ^{18}O - and $^{18}\text{O}_2$ -phosphorylation (Fig. 5B vs. Fig. 5A). The ratio of $^{18}\text{O}_x$ -phosphorylated ($x = 1$ and 2) to unlabeled phosphorylated metabolites were only 0.07. In contrast, when inorganic $^{18}\text{O}_4$ -phosphate, pH 7.4, buffer was added to the blood incubation, much greater amounts of ^{18}O -incorporated phosphorylation were observed (Fig. 5C vs. Fig. 5B vs. Fig. 5A), with the ratio of $^{18}\text{O}_x$ -phosphorylated ($x = 1, 2$, and 3) to unlabeled phosphorylated metabolites at 0.87 ± 0.03 (mean $\pm$ S.D. of triplicate incubations). Observed accurate masses of $^{18}\text{O}_x$ -phosphorylated metabolite ions ($x = 1, 2$, and 3) are all within 2 ppm error of calculated exact masses (Supplemental Table 1). When high concentration ^{18}O -water was present in the mouse blood incubation, ^{18}O - and $^{18}\text{O}_2$ -incorporated phosphorylation of AZ2381 occurred (Fig. 5D). The ratio of $^{18}\text{O}_x$ -phosphorylated ($x = 1$ and 2): unlabeled phosphorylated metabolites in the presence of the ^{18}O -water was 0.29 ± 0.004 (mean $\pm$ S.D. of triplicate incubations). It is important to note that additional oxygen-exchange tests on 2 μ M synthetic standard of phosphorylated-AZ2318 with approximately 6 mM $^{18}\text{O}_4$ -phosphate and 10 M ^{18}O -water in a PBS, pH 7.4, solution have indicated no chemical ^{18}O exchange to the phosphorylated AZ2381 standard after incubation at 37°C for 2 hours.

Rat blood also generated the phosphorylated metabolite of AZ2381, albeit at a lower yield than mouse blood (Fig. 6). The yield of phosphorylation product in the freshly collected rat blood was approximately one-fifth of that in freshly collected mouse blood under the same incubation conditions, on the basis of LC-UV peak areas and UV signal responses of the phosphorylated metabolite and the parent drug. The phosphorylation was also tested in commercially available

Wistar Hanover rat, Beagle dog, and human blood that were delivered to our laboratory overnight inside a cold pack. The phosphorylated metabolite turnover in dog and human blood was even lower than that in rat blood. However, the data are not presented here since the yield in overnight delivered rat blood was substantially lower than that in freshly collected rat blood at our laboratory; thus, the same would be assumed for the dog and human blood. The low-level phosphorylation in overnight-delivered rat, dog, and human blood was only detectable by LC-MS and was too low to be seen in LC-UV chromatograms.

Discussion

The fortuitous discovery of the phosphorylation of AZ2381 started with bioanalytical troubleshooting. After the surprising detection of phosphorylated AZ2381 in mouse plasma samples collected from the AZ2381-dosed animals, the possibility of analytical crosstalk from a common sulfation metabolite was considered. Nevertheless, the temporal profile and dose response of phosphorylated AZ2381 in mouse plasma after an oral dose of AZ2381 (Fig. 2) are consistent with the formation of a metabolite. If the sulfation of AZ2381 had occurred, the protonated sulfate metabolite would have the same unit mass as protonated phosphorylated AZ2381 ($[\text{M}+\text{H}]^+$ m/z 536, Supplemental Fig. 3). Both sulfate and phosphate of AZ2381 would give exactly the same fragment ion by the neutral loss of a H_2SO_4 and a H_3PO_4 molecule, respectively, ($[\text{M}+\text{H}]^+$ m/z 438, Supplemental Fig. 3). Moreover, the hypothetical sulfate could elute at approximately the same retention time as phosphorylated AZ2381 in the very short LC gradient used in bioanalysis, resulting in crosstalk. However, the sulfate would be readily differentiated from the phosphate by a high-resolution mass spectrometer, because their exact mass would differ by 9.5 mDa (relative error of 18 ppm, Supplemental Fig. 3). We anticipated proving this hypothesis of crosstalk but instead confirmed that it was a rare xenobiotic phosphorylation metabolite (Supplemental Fig. 1). As suggested by Mitchell (2016) in a recent review article, the possible misidentification of rare phosphate conjugates of xenobiotics as sulfate conjugates could have happened before the advent of modern LC-MS (such as the routine use of high-resolution MS in drug metabolism).

Heparin was used as the anticoagulant in this study to prevent chelation of the Mg^{2+} , which would occur with another commonly used anticoagulant, EDTA. The divalent metal ion Mg^{2+} is usually required in

Fig. 5. The molecular ion region of LC-MS spectra showing the phosphorylated or ^{18}O -phosphorylated metabolite produced after a 2-hour incubation of $10\ \mu\text{M}$ AZ2381 at 37°C in mouse blood (A), mouse blood with $10\ \text{mM}$ $\gamma\text{-}^{18}\text{O}_4\text{-ATP}$ added (B), mouse blood with approximately $6\ \text{mM}$ inorganic ^{18}O -phosphate buffer, pH 7.4, added (C), mouse blood with $10\ \text{M}$ ^{18}O -water added (D).

the kinase-mediated transfer of a phosphoryl group from an organic phosphate source to a kinase substrate (Matte et al., 1998; Harding et al., 2010). However, the addition of $10\ \text{mM}$ MgCl_2 to whole blood and RBC incubations did not enhance phosphorylation in our case (Figs. 3 and 4). It is unclear if sufficient Mg^{2+} ions were already present in whole blood and inside the isolated cells, or if a different type of divalent metal ion was involved in the phosphorylation. Nevertheless, the inhibitory effect of the metal chelator EDTA on phosphorylation (Figs. 3, 4, and 6) suggests that divalent metal ions are necessary for the phosphorylation reaction.

It is known that some enzymes in leukocytes can metabolize drug molecules, e.g., neutrophil myeloperoxidase (Uetrecht, 1994; Khan et al., 2016). The phosphorylation of the immunomodulatory drug fingolimod is catalyzed by sphingosine kinases (Billich et al., 2003) that are present in eukaryotic cells. The phosphorylation of AZ2381 occurred in both immune-competent CD-1 mouse and immune-deficient SCID and Nude mice, thus T and B lymphocytes that are absent from SCID and Nude mice are not responsible for the phosphorylation. Still, it was a bit surprising that remaining total leukocytes after the lysing of RBCs in the mouse blood gave virtually no phosphorylation of AZ2381 (Fig. 3E), as we were initially misled by a preliminary experiment with an isolated leukocyte fraction that was contaminated by some red blood cells (i.e., a low turnover of phosphorylated AZ2381 in the contaminated leukocytes fraction, data not shown). The incubations with collected intact RBCs further demonstrated that the phosphorylated metabolite was produced by mouse RBCs (Fig. 4). Isolation of leukocytes free of RBC contamination was also performed without lysing the red blood cells (*Materials and Methods*) to further verify that leukocytes were not responsible for the phosphorylation of AZ2381 (Fig. 4).

The intrinsic rate of the phosphorylation is probably higher than observed in the blood, because the reverse reaction of enzymatic hydrolysis of the organophosphate can occur. The yield of phosphorylation in total cellular fraction after removal of plasma from mouse blood was 2.6 times that in whole blood (Fig. 4). This increase in the yield of the phosphorylated metabolite could be rationalized by the removal of alkaline phosphatases and A-esterases present in plasma (Rosalki, 1994; Rooseboom et al., 2004; Parkinson et al., 2013). Both alkaline phosphatases and A-esterases would hydrolyze the organophosphate of the metabolite back to parent drug AZ2381. In the stability test of $2\ \mu\text{M}$ phosphorylated AZ2381 synthetic standard in freshly collected mouse plasma with heparin as anticoagulant at 37°C , a half-life ($t_{1/2}$) of approximately 4.5 hours was observed for the disappearance of phosphorylated AZ2381 standard, and concurrently AZ2381 was produced from phosphorylated AZ2381 (Supplemental Fig. 4).

The addition of ATP to the *in vitro* incubation with mouse blood or mouse RBCs did not increase the production of phosphorylated AZ2381 (Figs. 3 and 4). This could be explained in two different ways—either a sufficient amount of ATP is originally present in the blood and RBCs, or ATP is not the source of phosphoryl transfer in the RBC-mediated phosphorylation of AZ2381. It is known that ATP is the source of the phosphoryl group in most phosphorylation reactions (Matte et al., 1998; Cheek et al., 2002). However, other sources of phosphoryl groups may be used in some phosphorylations. In glycolysis (the sole source of metabolic energy in red blood cells), two inorganic phosphates (not ATP) are used to form two 1,3-bisphosphoglycerates, which are subsequently used by phosphoglycerate kinase to produce two ATP from two ADP in the payoff phase (Nelson and Cox, 2013). Additionally, two molecules of the high-energy phosphate compound phosphoenolpyruvate donate phosphoryl groups to two ADP molecules to form two ATP in the payoff phase of glycolysis (Nelson and Cox, 2013). Most glycolytic enzymes require Mg^{2+} for activity (Nelson and Cox, 2013).

Fig. 6. LC-MS accurate-mass extracted ion chromatograms comparing the formation of the phosphorylated metabolite following a 2-hour incubation of 10 μ M AZ2381 at 37°C in mouse blood with heparin as anticoagulant (A), rat blood with heparin as anticoagulant (B), the rat blood in the presence of 100 mM EDTA (C). Displayed are extracted ion chromatograms consisting of the same ions as in Fig. 3.

The level of ^{18}O -incorporation into the phosphorylation of AZ2381 in the presence of $\gamma\text{-}^{18}\text{O}_4\text{-ATP}$ was $<10\%$ that in the presence of inorganic $^{18}\text{O}_4\text{-phosphate}$ at a comparable concentration (Fig. 5B vs. Fig. 5C and data description in *Results*). Some degradation of $\gamma\text{-}^{18}\text{O}_4\text{-ATP}$ was evident from the presence of ^{18}O -ADP in the purchased $^{18}\text{O}_4$ -labeled ATP material as detected by the negative ion mass spectrum acquired in our laboratory (data not shown). Thus, inorganic $^{18}\text{O}_3\text{-phosphate}$ was presumably present as a degradation product in the $\gamma\text{-}^{18}\text{O}_4\text{-ATP}$ material used for the incubation. The abundance of ^{18}O -incorporated phosphorylated AZ2381 was in the order of $^{18}\text{O} > ^{18}\text{O}_2 > ^{18}\text{O}_3$ -phosphorylated when entirely ^{18}O -labeled $^{18}\text{O}_4$ -phosphate was added to the mouse blood incubation of AZ2381 (Fig. 5C). Also of note, when a large amount of ^{18}O -water (final concentration 10 M) was added to the mouse blood incubation, ^{18}O - and $^{18}\text{O}_2$ -phosphorylated AZ2381 metabolites were formed (Fig. 5D). Both observations could be rationalized by enzyme-mediated oxygen exchanges involving inorganic phosphate and water, e.g., oxygen exchange during ATP synthesis and reverse steps (Hackney, 1984). On the contrary, chemical exchange of oxygen isotope between dissolved phosphate and water would be extremely slow (Lecuyer et al., 1999), which is consistent with the observation that there was no chemical ^{18}O exchange from $^{18}\text{O}_4$ -phosphate or ^{18}O -water to the phosphorylated AZ2381 synthetic standard in our control experiment.

In summary, the present study has confirmed rare xenobiotic phosphorylation of a drug molecule that is not intended to be a kinase substrate. The fact that the phosphorylation is mediated by red blood cells and the phosphoryl transfer does not come from ATP may have made this case particularly unusual. However, a number of questions are yet to be addressed—whether the turnover of phosphorylated AZ2381 is lower rat blood than in mouse blood as the result of a lower formation rate or higher hydrolysis rate of phosphorylated AZ2381, what specific

enzyme in red blood cells is responsible for the phosphorylation, and what structural feature of AZ2381 triggers a phosphorylation that is usually intended for endogenous substances.

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Authorship Contributions

Participated in research design: Gu, Wen, Doig.
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Contributed new reagents or analytic tools: Zheng, Johannes.
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