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# Intrinsic electrophilicity of the 4-methylsulfonyl-2-pyridone scaffold in glucokinase activators: Role of glutathione-*S*-transferases and in vivo quantitation of a glutathione conjugate in rats

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ABSTRACT

Previous studies on the in vitro metabolism of 4-alkylsulfonyl-2-pyridone-based glucokinase activators revealed a facile, non-enzymatic displacement of the 4-alkylsulfonyl group by glutathione. In the present studies, a role for glutathione-*S*-transferases (GST) as catalysts in the desulfonylation reaction was demonstrated using a combination of human liver microsomes, human liver cytosol and human GSTs. The identification of a glutathione conjugate in circulation following intravenous administration of a candidate 4-methylsulfonyl-2-pyridone to rats confirmed the relevance of the in vitro findings.



Innately electrophilic compounds represent a significant liability in drug discovery and development because they possess chemical reactivity similar to affinity labeling agents (e.g., N-substituted maleimides and/or alkyl halides); such compounds can alkylate proteins, DNA and/or the endogenous antioxidant glutathione (GSH), leading to toxicological outcomes.[1–3] Consequently, electrophilic functional groups (e.g., alkyl halides, Michael acceptors, etc.) are generally avoided in drug design.[4] Despite the necessary diligence, there have been reports in the medicinal chemistry literature on seemingly 'chemically inert' compounds, which are prone to nucleophilic displacement by GSH under non-enzymatic conditions (pH 7.4, phosphate buffer, 37 °C) and/or enzyme-catalyzed conditions.[5–8] In the case of enzyme-assisted reactions, GSH conjugation to electrophilic centers is mediated by microsomal, cytosolic and/or mitochondrial glutathione-*S*-transferase (GST).[9–11]

In the course of efforts to optimize 4-sulfonyl-2-pyridone-based glucokinase activators as anti-diabetic agents, a unique metabolic liability of the 4-sulfonyl-2-pyridone scaffold in the lead compound **1** was identified wherein the heterocycle readily adducted to GSH in phosphate buffer to form conjugate **2** (Fig. 1).[12] The mechanism involves nucleophilic attack by the thiolate anion of GSH at the electrophilic center on the pyridone ring to yield the negatively charged Meisenheimer complex followed by elimination of the methylsulfonyl group (Fig. 1). Analogs **3**–**7** (Fig. 1) with increasing steric bulk either directly on the sulfone or in the adjacent position on the ring also reacted readily with GSH suggesting that chemical reactivity with GSH was not sensitive to steric factors.

While the results from our previous work established the electrophilic nature of **1**, the role of GST isozymes in catalyzing the nucleophilic displacement reaction and the relevance of this overall liability in vivo remained unclear. In the present work, we undertook a side-by-side characterization of the in vitro reactivity of **1** and related 2-pyridone analogs **24**–**27** (Scheme 2) with GSH under non-enzymatic (phosphate buffer, pH 7.4, 37 °C) and enzymatic (human liver microsomes, human liver cytosol and human GST enzymes) conditions. In vivo studies on **1** were also conducted in the rat to quantitatively assess the presence of **2** in circulation (plasma and bile). The collective findings are summarized, herein.

To enable analytical quantitation of GSH conjugate **2** in biological matrices, an authentic standard was synthesized via the base mediated reaction of GSH with **1** in 63% yield (Scheme 1).[13]

Substitutedpyridones **24–27** were synthesized via a pyridone N-alkylation strategy depicted in Scheme 2. The preparation of requisite 2-pyridones (compounds **9**, **12** and **16**) that were utilized in the N-alkylation reaction is also shown in Scheme 2.

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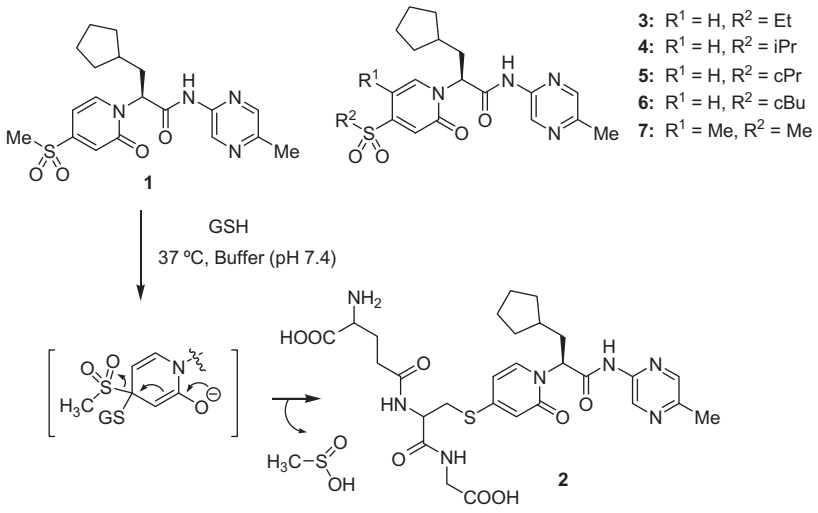
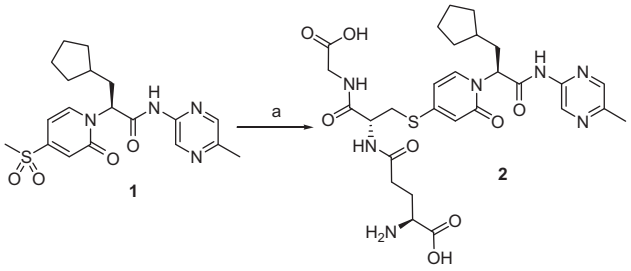


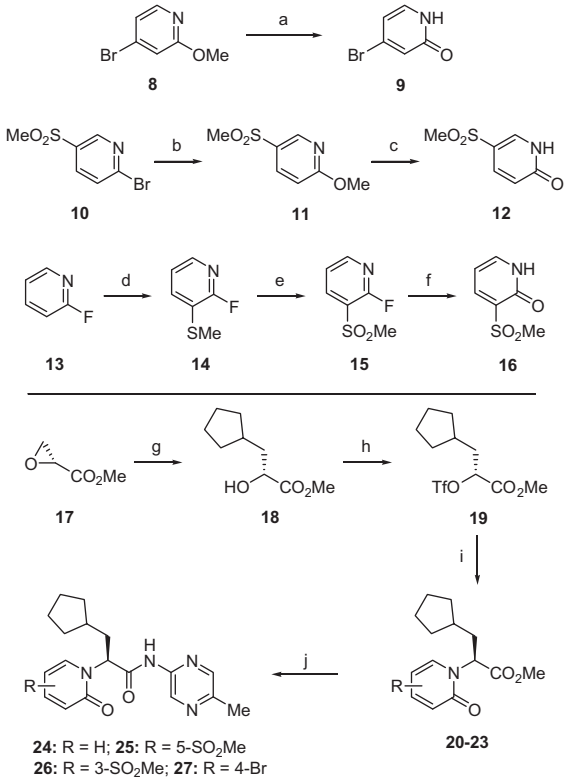

**Figure 1.** Chemical reactivity of 4-sulfonyl-2-pyridone-based glucokinase activator **1** with glutathione.




**Scheme 1.** Reagents and conditions: (a) L-glutathione (1.7 equiv), $Et_3N$ (3.0 equiv), MeOH, 55 °C, 72 h, 63%.

2-methoxyl-5-methylsulfonylpyridine (**11**) was prepared from 2-bromo-5-methylsulfonylpyridine (**10**)[14] via a Pd-catalyzed coupling reaction.[15] O-Demethylation of **8** or **11** with iodotrimethyl silane (generated in situ from chlorotrimethylsilane and sodium iodide) afforded 4-bromo-2-pyridone (**9**) and 5-methylsulfonyl-2-pyridone (**12**), respectively. Thioanisole **14** was synthesized from **13** through a base mediated reaction with methylthiocyanate. S-Oxidation of **14** in the presence of mCPBA generated sulfone **15**. Nucleophilic displacement of the 2-fluoro group in **15** by water afforded 3-methylsulfonyl-2-pyridone (**16**). Hydroxymethyl ester **18** was prepared via nucleophilic opening of epoxide **17**.[16] Reaction of **18** with trifluoromethanesulfonic anhydride in the presence of 2,6-lutidine generated triflate **19**. Alkylation of pyridone and its derivatives (compounds **9**, **12**, **16**) was accomplished via deprotonation with lithium bis(trimethylsilyl)amide followed by reaction with **19** to afford the N-alkylated derivatives (**20**–**23**). Trimethylaluminum-mediated transamidation of ester derivatives **20**–**23** with 2-amino-5-picoline provided the title compounds **24**–**27**, respectively.

The in vivo formation of **2** was examined in rats after administration of a single intravenous bolus dose (5 mg/kg) of **1**. A representative extracted ion chromatogram of plasma from rats administered with **1** is shown in Figure 2. The retention time ($R_t$) and mass spectral characteristics ($MH^+ = 632$, $m/z + H^+ = 523$, 394) of the peak that eluted at 1.8 min were identical to those observed for the synthetic standard of **2**. Figure 3 depicts the time course profile of mean rat plasma and biliary concentrations of **1** and GSH conjugate **2**.[17] The rapid appearance of **2** in rat plasma (as early as 5 min with peak total plasma concentrations of ~8.0 μM) indicates the efficiency of reaction between **1** and GSH




**Scheme 2.** Reagents and conditions: (a) TMSCl (1.5 equiv), NaI (1.5 equiv), MeCN, 23 °C, 5 h, 29%; (b) $Pd_2(dba)_3$ (0.005 equiv), bippyphos (0.02 equiv), KOH (1.5 equiv), MeOH, reflux, 16 h, 91%; (c) TMSCl (1.8 equiv), NaI (1.8 equiv), MeCN, 23 °C, 4 h, 100%; (d) LDA (1.0 equiv), MeSCN (1.0 equiv), THF, −78 to 0 °C, 2.5 h, 92%; (e) *m*CPBA (2.5 equiv), DCM, 0 °C, 2 h, 94%; (f) $H_2O$, cat. acid; (g) $Li_2CuCl_4$ (0.1 equiv prepared from $CuCl_2$ [0.1 equiv], LiCl [0.2 equiv], THF), cyclopentylmagnesium bromide (2.0 M in $Et_2O$, 1.1 equiv), THF, −78 to −50 °C, 30 min, 70%; (h) $Tf_2O$ (1.8 equiv), 2,6-lutidine (2.1 equiv), DCM, 0 °C, 30 min; (i) (1) pyridone (1.0 equiv), LiHMDS (1.0 M in THF, 0.9 equiv), THF, 23 °C, 45 min, (2) **19** (1 equiv), THF, 23 °C, 2 h, 44–85%; (j) 5-methylpyrazin-2-amine (2–4 equiv), $Me_3Al$ (2.0 M in toluene, 2.2–4.4 equiv), 1,2-DCE, 80 °C, 1–2 d, 53–71%.

in rats. GSH conjugate **2** appeared to be the predominant metabolite of **1** in circulation; oxidative metabolites (mono-hydroxylation on the cyclopentyl and methylpyrazine ring systems) represented a very minor component of drug-related material in circulation. The in vivo observation is consistent with the low oxidative

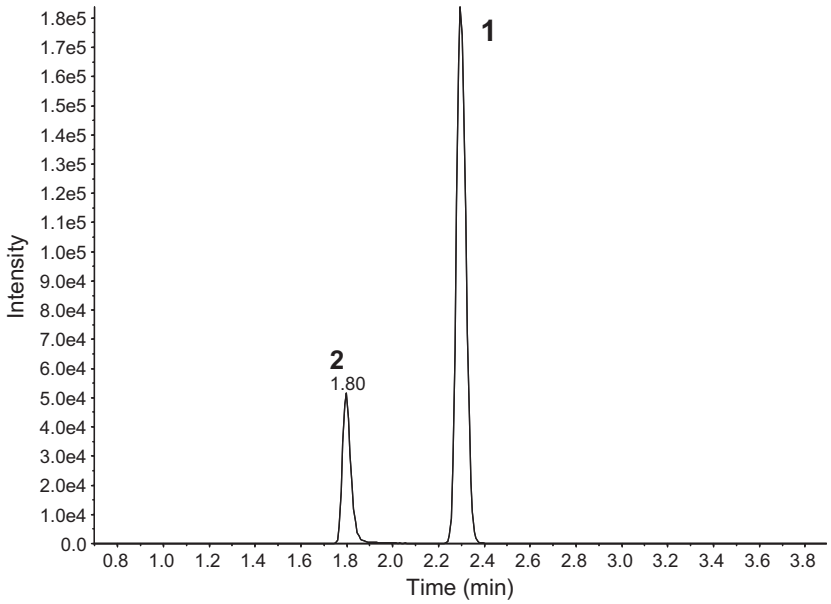



**Figure 2.** Extracted ion chromatogram of pooled rat plasma following a single intravenous bolus dose of **1** at 5 mg/kg.

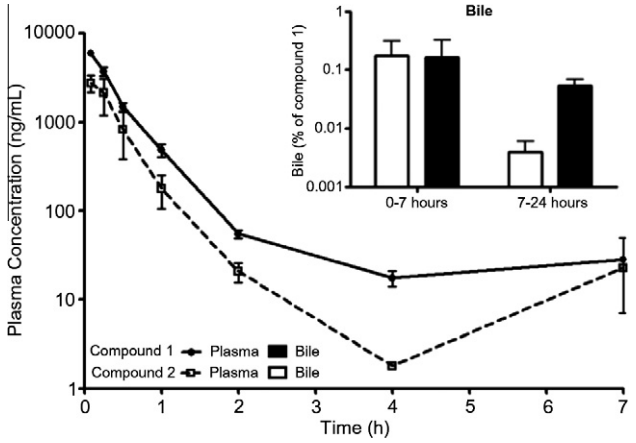



**Figure 3.** Mean concentration versus time profile of **1** and its GSH conjugate **2** in plasma and bile from rat administered an intravenous bolus dose of **1** (5 mgx/kg).

turnover of **1** in rat liver microsomes and rat hepatocytes.[12] While not directly assessed in this in vivo study, it is possible that repeated dosing of **1** could deplete the endogenous pool of GSH in rats leading to toxicity in a similar fashion to that observed with the anti-inflammatory agent acetaminophen.[18,19]

In order to examine whether GST enzymes catalyzed the nucleophilic displacement reaction, the formation of **2** was monitored in side-by-side in vitro incubations of **1** (10 μM) and GSH (5 mM) in phosphate buffer (pH 7.4), human liver microsomes, human liver cytosol and human placental GSTs.[20] In each instance, the UV chromatogram ($\lambda$ = 300 nm) revealed the formation of a single metabolite ($R_t$ ~16 min (Fig. 4). The $R_t$ and mass spectral characteristics of this metabolite were identical with those of the synthetic standard of **2**. The formation of **2** in microsomes that lacked NADPH (Fig. 4, panel C) confirmed that prior metabolic activation to a reactive species by cytochrome P450 isoforms was not required for the nucleophilic displacement reaction. In incubations with human placental GSTs, a quantitative conversion of **1** to **2** was observed (Fig. 4, panel E). Furthermore, the detection of **2** in GSH-supplemented microsomes and cytosol (Fig. 4, panels B–D) suggested that both microsomal and cytosolic GST enzymes were capable of converting **1** to **2**.

Under the present experimental conditions, the possibility of direct Michael addition of GSH on the 2-pyridone moiety was examined with **24** (R = H) and the methylsulfone regioisomers of **1**, that is, compounds **25** (R = 5-$SO_2$Me) and **26** (R = 3-$SO_2$Me). Likewise, the 4-bromo-2-pyridone derivative **27**, which is a harder, less electrophilic substrate (relative to **1**) was also included in this analysis. Following incubations of compounds **24**–**26** and GSH in buffer, microsomes, cytosol and/or GSTs, no GSH conjugates derived from Michael addition were observed. Likewise, no nucleophilic displacement of the 4-bromo substituent by GSH was observed in incubations of **27** and GSH in phosphate buffer or human liver microsomes (in the absence and/or presence of NADPH co-factor) (Fig. 5, panels A–C). In human liver microsomal incubations fortified with NADPH, only metabolites derived from P450-catalyzed oxidation on the cyclopentylmethylene and pyrazinylmethyl regions in **27** were discerned (Fig. 5, panel B). In contrast with the findings in liver microsomes, incubations of **27** and GSH in human liver cytosol and human GSTs led to the formation of a single metabolite (Fig. 5, panels D and E), which was unambiguously proven to be GSH conjugate **2** by virtue of identical LC $R_t$ and mass spectral characteristics with those of the authentic standard. Thus, while **27** was stable towards reaction with GSH under non-enzymatic conditions, a facile displacement reaction occurred upon inclusion of cytosolic fraction and/or GST enzymes. Lack of analogous reactivity in liver microsomes suggests that GSH displacement of the 4-bromo substituent in **27** (see Fig. 5) is exquisitely mediated by cytosolic GST enzymes.

As metabolic activation is not a prerequisite to generation of a transient reactive species for 4-substituted-2-pyridones, it would be expected that the inherent electrophilicity and chemical softness parameters of these compounds would adequately predict their relative reactivity with GSH. Indeed, 4-bromopyridone **27** was calculated to be a harder, less electrophilic substrate overall when compared to the softer, more electrophilic 4-methylsulfonylpyridone derivative **1** (Fig. 6);[21,22] this corresponded well with the observed reactivity of GSH with these compounds under non-enzymatic conditions. However, caution should be exercised when applying the substrate electrophilicity number on an enzymatic system such as GST, because of the synergetic interactions between the enzyme and the substrate that stabilize the transition state intermediate.

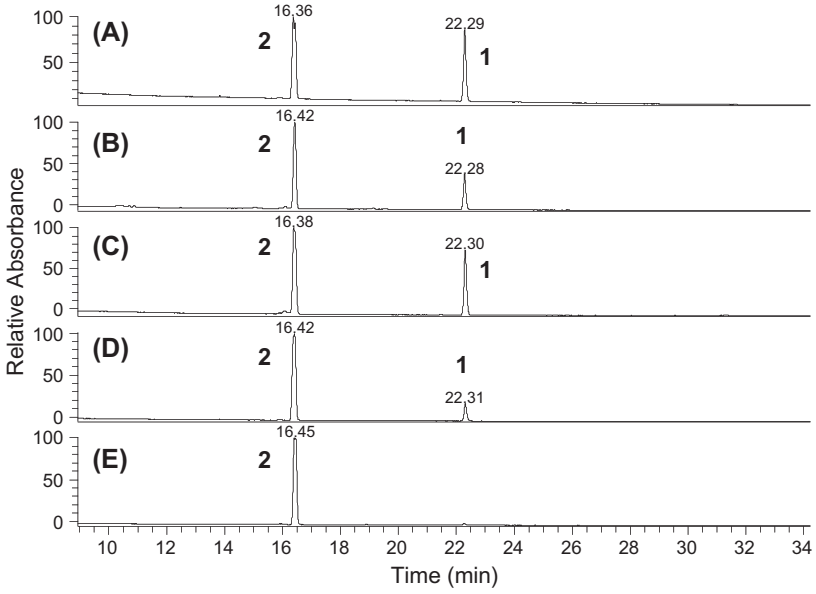



**Figure 4.** UV chromatograms of incubations of **1** (10 μM) in: phosphate buffer (pH 7.4) and GSH (5 mM) (panel A), NADPH-supplemented human liver microsomes and GSH (5 mM) (panel B), human liver microsomes and GSH (panel C), human liver cytosol and GSH (5 mM), (panel D), and human placental GST and GSH (5 mM) (panel E). All incubations were conducted at 37 °C for 60 min in a shaking water bath.

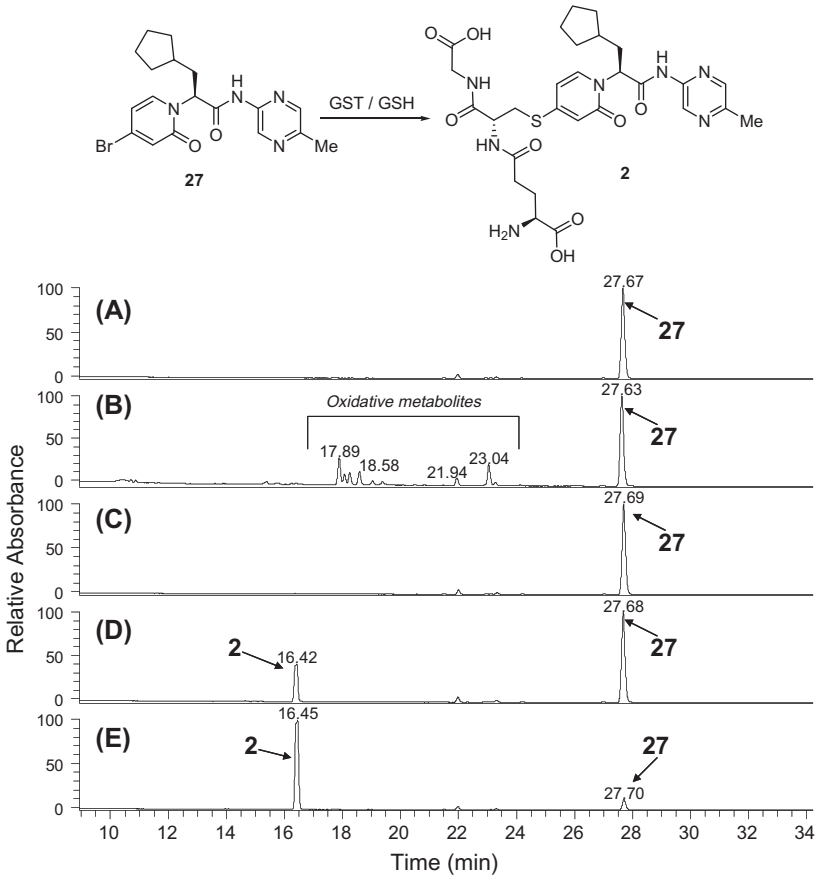



**Figure 5.** UV chromatograms of incubations of **27** (10 μM) in: phosphate buffer (pH 7.4) and GSH (5 mM) (panel A), NADPH-supplemented human liver microsomes and GSH (5 mM) (panel B), human liver microsomes and GSH (panel C), human liver cytosol and GSH (5 mM) (panel D), and human placental GST and GSH (5 mM) (panel E). All incubations were conducted at 37 °C for 60 min in a shaking water bath.

Overall, the results described herein provide a cautionary note to drug discovery teams, which solely rely on reactive metabolite trapping in liver microsomes as means of evaluating bioactivation potential of new chemical entities. Based on the absence of a GSH adduct(s) under non-enzymatic conditions and in human liver microsomes (±NADPH), the 4-bromo-2-pyridone derivative **27**

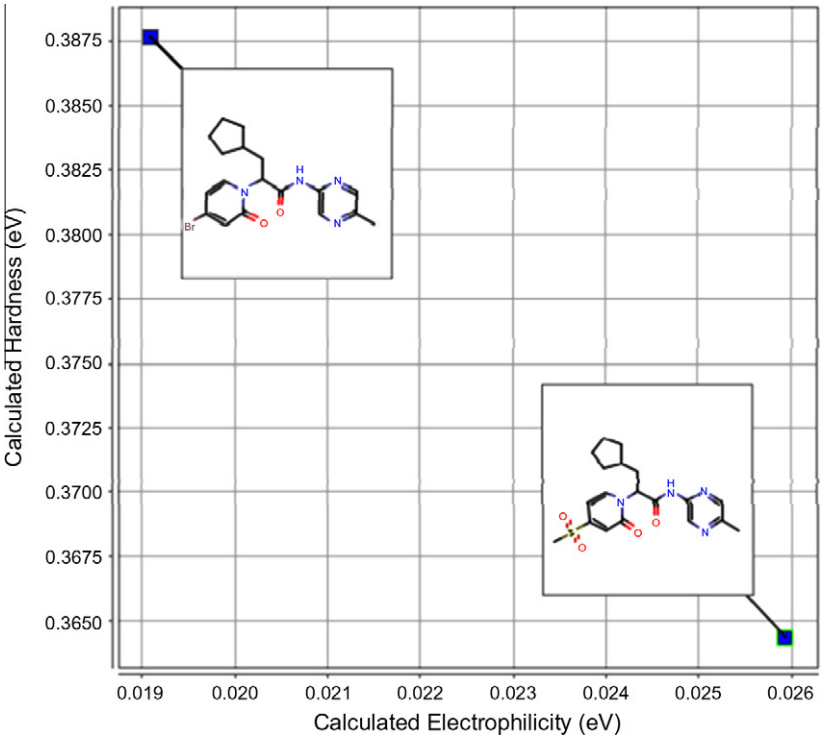



**Figure 6.** Electrophilicity prediction of **1** and **27**.

would have passed the reactive metabolite screen with flying colors. Metabolism vectors other than liver microsomes need to be taken into consideration when evaluating bioactivation and/or innate electrophilicity of lead chemical matter.

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13. Procedure for the synthesis of GSH conjugate **2**. Sulfone **1** (80 mg, 0.2 mmol, prepared according to Ref. 12) and L-GSH (102 mg, 0.3 mmol) were combined in 1 mL of MeOH in a septum capped screw–cap vial. The mixture was placed under argon and anhydrous $Et_3N$ (85 μL, 0.6 mmol) was added via syringe. The clear orange solution was heated at 55 °C for 3 days. The mixture was then cooled, neutralized with 300 μL of 1 M HCl, and chromatographed on a 12 g reverse-phase C18 column using a water–MeCN gradient. Fractions containing the desired product were concentrated to a semisolid residue, which was azeotropically dried with MeCN at reflux, then cooled and concentrated to afford **2** as a white powder (79 mg, 63%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ ppm 1.16 (dd, $J$ = 11.80, 7.71 Hz, 2H) 1.23–1.33 (m, 2H) 1.41–1.57 (m, 2H) 1.57–1.70 (m, 3H) 1.70–1.84 (m, 2H) 2.05–2.24 (m, 3H) 2.49 (s, 3H) 2.52–2.59 (m, 1H) 3.24–3.29 (m, 2H) 3.49–3.59 (m, 1H) 3.62–3.77 (m, 1H) 3.84–3.95 (m, 1H) 4.73 (br s, 1H) 5.72–5.85 (m, 1H) 6.39 (d, $J$ = 7.41 Hz, 1H) 6.50 (br s, 1H) 7.68 (d, $J$ = 7.41 Hz, 1H) 8.27 (s, 1H) 9.14 (s, 1H). LC–MS: 632 ($MH^+$).
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17. All animal care and in vivo procedures conducted were in accordance with guidelines of the Pfizer Animal Care and Use Committee. Compound **1** (5 mg/kg) was administered intravenously via the jugular vein cannula of bile-duct exteriorized male Wistar-Han rats (0.31–0.36 kg, $n$ = 3). Compound **1** was formulated in DMSO/propylene glycol/50 mM tris buffer (10:50:40, v/v/v). After dosing, serial blood samples were collected at appropriate times, and plasma was obtained via centrifugation and kept frozen at −20 °C until analysis. Bile samples (0–7.0 and 7.0–24 h) were also collected after iv administration to rats. Liquid-chromatography tandem mass spectrometry (LC–MS/MS) was performed on a Sciex API4000 system equipped with a turbo-ionspray source (Applied Biosystems, Foster City, CA, USA) operated in positive ion mode. HPLC analysis was conducted on Shimadzu 10ADvp Binary HPLC system (Shimadzu Scientific Instruments Columbia, MD, USA) with a CTC-PAL (Thermo Scientific, Franklin, MA, USA) as the autosampler. Chromatographic separations were performed on a Phenomenex Synergy Max-RP 2 × 50 mm 4 μ HPLC column (Torrance, CA, USA) using mobile phase A (10 mM ammonium acetate and 1% isopropyl alcohol in water) and mobile phase B (acetonitrile). A linear gradient from 5% B to 95% B over 1.5 min with the flow rate of 0.3 ml/min was performed to elute **1**, **2** and the internal standard. Analyst (version 1.4.1, Applied Biosystems, Foster City, CA, USA) was employed to control the instrument operation and acquire data in multiple reaction monitoring mode. The ion transition for **1** and **2** were 405→296 and 632→523, respectively. The dynamic range of the assay ranged from 5 ng/ml to 5000 ng/ml using linear regression with a weighting of $1/x^2$.
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20. Test compounds (10 μM) were incubated in phosphate buffer (0.1 M, pH 7.4) in the presence or absence of GSH (5 mM) for 60 min at 37 °C. Separately, test compounds (10 μM) were also incubated with human liver microsomes (2.0 mg/ml) or human liver cytosol (2.0 mg/ml) or human placental GST (2.6 U/ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing $MgCl_2$ (3.3 mM) and NADPH (1.3 mM) in the presence of GSH (5 mM). In the case of microsomal and cytosol incubations, reactions were initiated with the addition of the biological matrix (microsomes or cytosol). Control incubations were run in parallel in the absence of NADPH and/or GSH. All incubations were

conducted in a shaking water bath maintained at 37 °C open to the air. After 60 min, the incubations were terminated by addition of ice-cold acetonitrile containing 0.1% formic acid, mixed vigorously, and the precipitated materials were removed by spinning in a centrifuge (3000*g*) for 5 min. Aliquots of the supernatants were analyzed for metabolite formation by LC–MS/MS. The HPLC system consisted of an Accela quaternary solvent delivery pump and autoinjector, a Surveyor PDA Plus photodiode array detector (Thermo Electron Corporation, Waltham, MA). Chromatography was performed on a Phenomenex, Synergy RP column, 150 × 4.6 mm, 5 μm (Phenomenex, Torrance, CA). LC analysis was performed at a constant flow rate of 1000 μl/min using a binary solvent system: Solvent A, 5 mM ammonium formate buffer (pH ~3.0) with 0.1% formic acid and Solvent B, acetonitrile. The initial HPLC gradient system was held at 5% B for 3 min and linearly increased to 80% B in 35 min, followed by a return to initial conditions for column re-equilibration. Post-column flow passed through the PDA detector to provide UV (λ = 200–400 nm) detection prior to being split to the mass spectrometer such that mobile phase was introduced into the electrospray source at a rate of 50 μl/min. The LC system was interfaced to a Thermo Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Xcalibur software version 2.0 was used to control the HPLC/MS system. Mass spectroscopy analyses were carried out in the positive ion mode using full-scan MS with a mass range of 100–1000 Da. Full scan data and data-dependent MS/MS acquisition on the two most intense ions were collected at 15,000 resolution. All experimental data were acquired using external calibration prior to data acquisition.

21. The measure of a ligand's electrophilicity is defined as Eq. 1. (Ref. 22):

$$\omega = \frac{\chi^2}{2\eta} \tag{1}$$

where $\chi$ is electronegativity (Eq. 2), and $\eta$ is hardness (Eq. 3)

$$\chi = -\frac{E_{\mathrm{HOME}} + E_{\mathrm{LUMO}}}{2} \tag{2}$$

$$\eta = E_{\mathrm{LUMO}} - E_{\mathrm{HOMO}} \tag{3}$$

In our implementation, an input chemical structure was first neutralized and then minimized with MacroModel module in Maestro software package (Maestro 9.0, Schrodinger LLC, NYC, NY). After that $E_{\mathrm{HOMO}}$ and $E_{\mathrm{LUMO}}$ energies were calculated with Jaguar module using HF/3-21G* level of theory.

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