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Metabolic activation of *N*-thiazol-2-yl benzamide as glucokinase activators: Impacts of glutathione trapping on covalent binding

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ABSTRACT

Glucokinase activators (GKAs) are currently under investigation as potential antidiabetic agents by many pharmaceutical companies. Most of GKAs reported previously possess *N*-aminothiazol-2-yl amide moiety in their structures because the aminothiazole moiety interacts with glucokinase (GK) and shows strong GK activation. During the development of *N*-aminothiazol-2-yl amide derivatives, we identified a bioactivation and metabolic liability of 2-aminothizole substructure of GKA **3** by assessing covalent binding, metabolites in liver microsomes and glutathione (GSH) trap assay.

GK, a member of the hexokinase family, is expressed in the liver and in pancreatic β-cells.[1] This enzyme catalyzes the key initial step for glucose metabolism, that is, phosphorylation of glucose to glucose 6-phosphate. In the liver, GK promotes glycogen synthesis, whilst it enhances insulin secretion from pancreatic β-cells.[2–4] Therefore, GKAs can be expected to function as an anti-hyperglycemic drug, by both increasing glucose uptake in the liver and the potentiation of insulin secretion from pancreatic β-cells.[5–8] As a result of promising preclinical data, many pharmaceutical companies have actively pursued this target aiming at the development of GKAs.[9–19] Of these, several companies including Roche, Astra-Zeneca, and OSI/Prosidion have advanced into clinical studies.[10,12]

Most of the GKAs reported previously possess the *N*-aminothiazol-2-yl amide moiety in their structures because the aminothiazole substructure interacts with Arg63 of GK and shows strong GK activation.[20] We have also developed and reported 2-aminothiazole-2-yl-containing benzamides as GKAs exemplified by compounds **1–3** (Fig. 1).[13–15] Compound **3**, for instance, showed potent GK activation and glucose lowering effects in diabetic mice models.[15] Thus, the GKA **3** was evaluated further to establish its potential for biochemical activation and its metabolic profiles.

As a part of drug discovery and development, the metabolic profiles of new drug candidates must be characterized. Drug metabolites are the products of enzymatic modifications such as oxidation or conjugate formation. Intermediates in the metabolic reactions, or the products themselves, may be reactive and give rise to covalent protein binding. Evaluation of this is important since covalent protein modification may lead to unwanted toxicities and idiosyncratic reactions in human.[21]

In brief, the potential of drug candidates to cause covalent binding is first evaluated in vitro by incubation of a radiolabeled analog in the presence of rat and human liver microsomes under oxidative conditions. In both cases, formation of covalent adducts with protein is determined by successive washing of protein pellets using either Brandel harvester technique or centrifugation-based methods.[22–24] A target value of 50 pmol-equiv/mg protein at 1 h for the in vitro assay was proposed by Evans et al., considering these

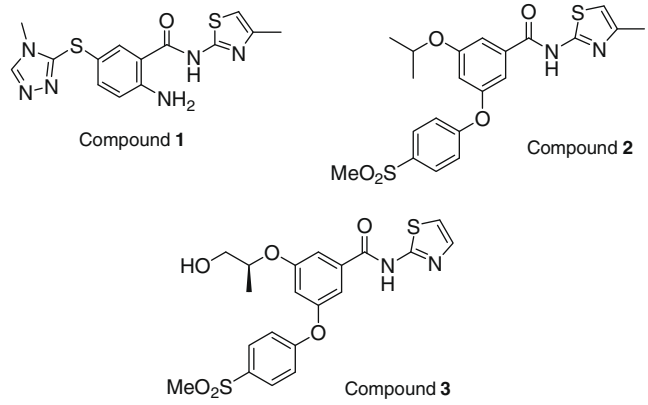

Figure 1. Structures of GK activators **1–3**.

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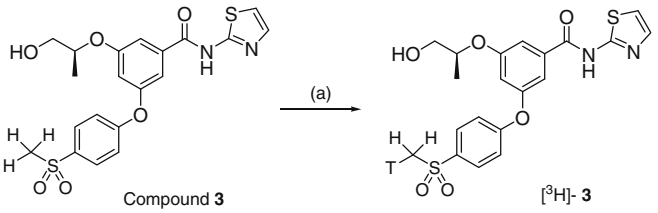

Scheme 1. Preparing of tritium labeled **3**. Reagents and conditions: (a) (1) T_2O–H_2O (5 Ci/mL), DBU, THF, specific activity: 102.2 mCi/mmol, purity: >99.4%.

values are approximately 10-fold over the background of the assays and represent 1/20th of binding obtained for known hepatotoxins.[21]

Table 1
Covalent binding data of compound **3** in human and rat liver microsomes

NADPH	Covalent binding[a]	
	Human MS (HM)	Rat MS (RM)
(+)	715 ± 2	378 ± 23
(−)	10 + 9	0 + 0

[a] Values reported as pmol-equiv binding/mg protein at 1 h.

This paper herein describes the in vitro covalent binding results that were observed during the course of evaluating a GKA **3** for the treatment of type-2 diabetes. Work was first performed to identify what reactive species was responsible for protein labeling, using a glutathione (GSH) as trapping agent.

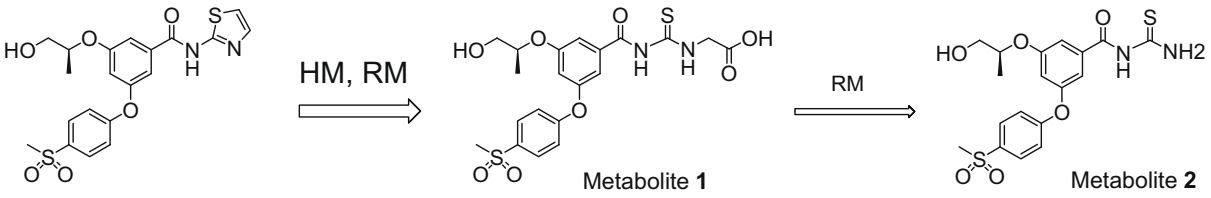

Figure 2. Major metabolites of **3** in human and rat microsomes. HM: Human microsomes, RM: Rat microsomes.

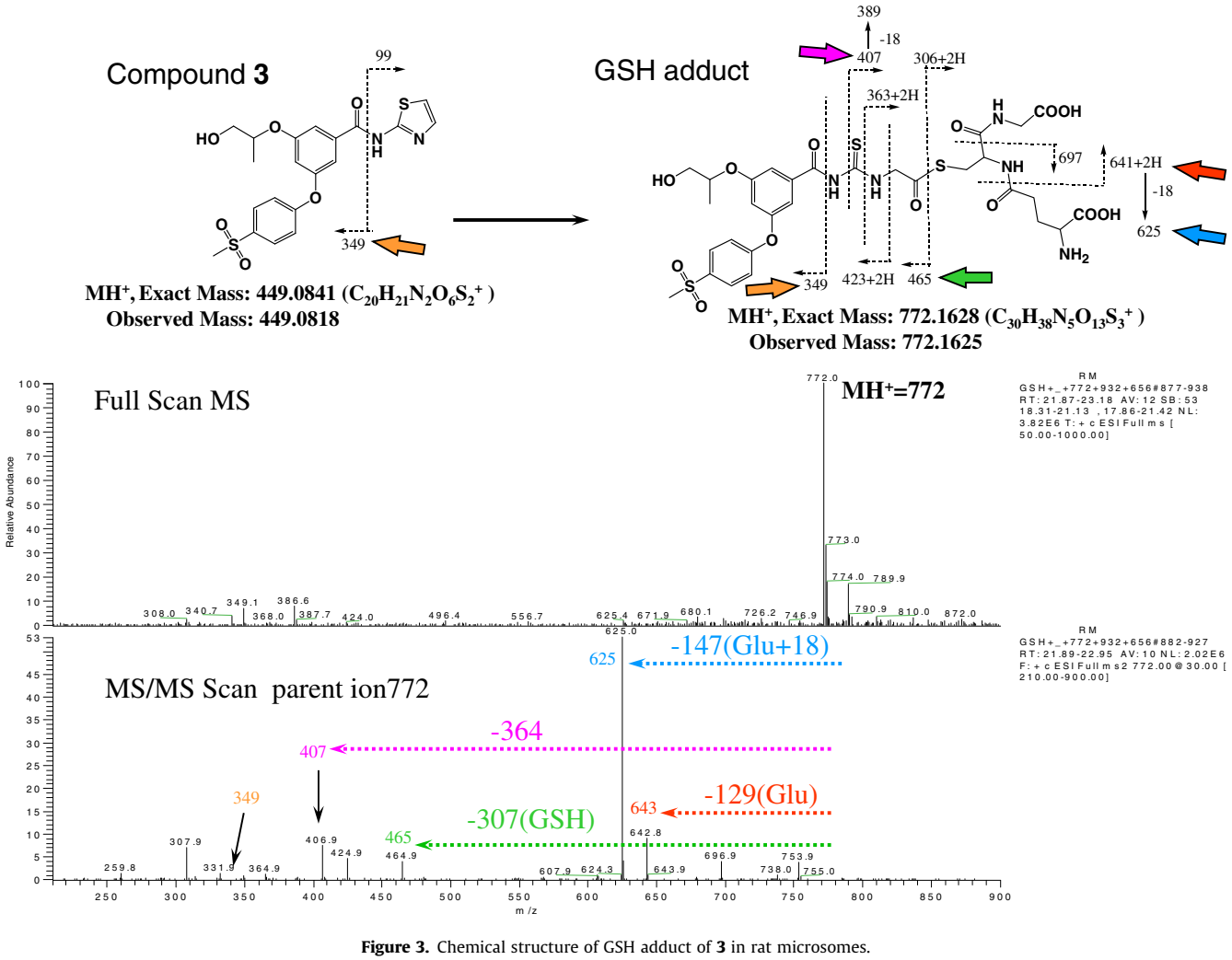

Figure 3. Chemical structure of GSH adduct of **3** in rat microsomes.

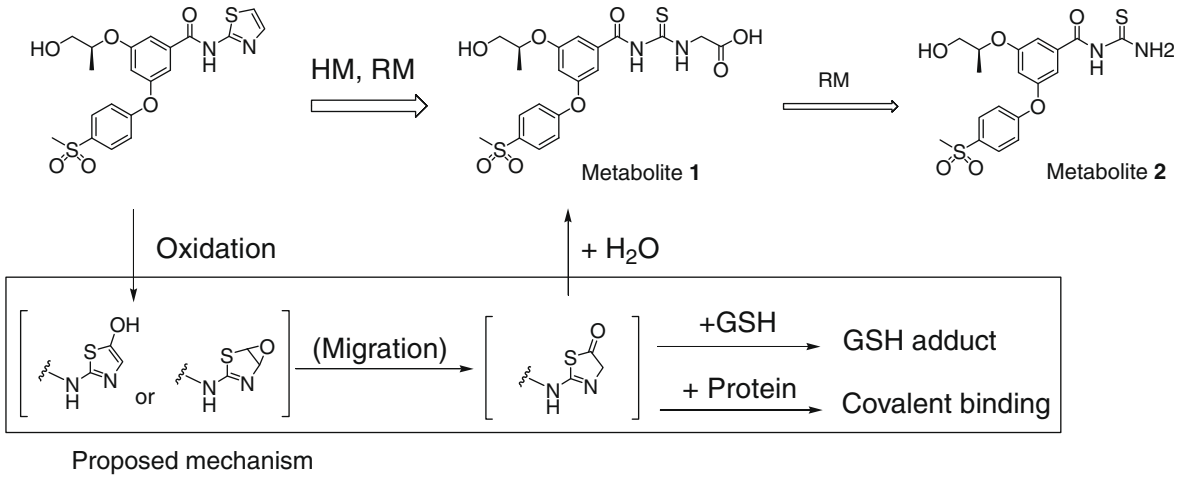

Figure 4. Proposed mechanism leading to GSH adduct formation and covalent binding.

Further investigation of compound **3** was initiated to assess its potential to cause in vitro protein covalent binding. Radio labeled compound **3**, tritiated at the methylsulfone moiety, was synthesized as shown in Scheme 1.[25] A base-catalyzed exchange reaction was conducted with tritiated water and DBU to afford tritium labeled compound **3** giving specific radioactivity of 102 mCi/mmol.[26,27]

Using this labeled compound, in vitro liver microsomal covalent binding experiments were performed, as described previously.[28] As shown in Table 1, tritium labeled **3** led to a significant increase in protein labeling in both rat and human liver microsomes in an NADPH dependent manner. Due to the potential for extensive covalent binding through bioactivation, further evaluation of this compound was discontinued.

In vitro incubations of **3** were repeated using unlabeled material and analyzed by high performance liquid chromatography coupled to mass spectrometric detectors (HPLC–MS/MS).[29] The major metabolite of **3** was identified to be the ring-opened metabolite **1** in both human and rat microsomes (Fig. 2). In rat microsomes, the thiourea metabolite **2** was also observed; this is considered to be a toxic metabolite.[30–32] To investigate the mechanism of formation of metabolites, GSH trapping assay was conducted.

Chemical trapping agents such as GSH, cyanide, semicarbazide or methoxylamine have been used in the past to identify reactive metabolites.[21,23] The potential of GSH, a soft nucleophile, to trap the reactive metabolite of **3** was assessed by repeating the rat in vitro liver microsomal covalent binding assay in the presence or absence of physiologically relevant concentrations of GSH (5 mM). A fivefold decrease in protein labeling was observed in the presence of GSH (from 272 to 50 pmol-equiv/mg protein at 1 h) and a concomitant formation of new metabolite showing a +323 u mass shift relative to **3**, characteristic for oxidative oxygen atom (+16) and glutathione adduct (+307). The GSH adduct was analyzed by HPLC–MS/MS and identified to be a ring-opening thioester as shown in Figure 3.

As observed for **3**, addition of millimolar quantities of GSH led to decreases in the extent of in vitro covalent binding in parallel with increased formation of the corresponding GSH adduct. A mechanism implicating CYP-catalyzed oxidation and subsequent migration of oxygen atom, leading to formation of the thio-lactone ring, was postulated.[31] This metabolic pathway would account for either the covalent binding or the GSH adduct (Fig. 4).

In preliminary experiments, it was found that the levels of covalent binding of substituted thiazolyl derivatives of compound **3** were lower than those of **3**. 2–5-fold decreases in covalent protein labeling were observed in testing 4-hydroxymethyl-1,3-thiazol-2-yl amide derivative and 5-methyl-1,3-thiazol-2-yl amide derivative (191[113] and 173[268] pmol-equiv/mg protein in human[rat] microsomes, respectively). It was suggested that introduction of substituents on the thiazole ring would be effective to reduce the CYP-catalyzed oxidation.

In conclusion, GKA **3** containing a 2-aminothiazole substructure was shown to cause protein labeling in presence of rat and human liver microsomes under oxidative conditions. Presence of GSH as a trapping agent led to reduction of in vitro covalent binding and to concomitant formation of a glutathione adduct to the thiazole ring, suggesting the potential implication of a reactive thio-lactone ring.

Comparison studies using not only other aminothiazole derivatives but the other heteroaromatics are underway.

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26. To a solution of compound **3** (1.0 mg) in THF (0.5 mL) containing DBU (5 μl) was added tritiated water (200 μl, 5 Ci/mL). The mixture was stirred in a closed vial at room temperature for overnight. To the mixture was added ethyl acetate (2 mL) and 1 N HCl (1 mL) containing saturated brine. The mixture was partitioned, dried and concentrated. The crude residue was purified by preparative HPLC to afford tritiated compound **3** (yield: ca. 0.7 mg, specific activity: 102.2 mCi/mmol, total activity: 165.8 μCi, purity: >99.4% (radio HPLC, 210 nm).
27. We have shown experimentally in house by radiometric HPLC that no back exchange of tritium at methyl sulfone moiety and no metabolism at the site of tritiation was taking place.
28. Compound **3** (10 μM, 50 mCi/mmol) was added to a phosphate-buffered solution (100 mM; pH 7.4) containing 1 mg/mL of rat or human liver microsomes. Following a 15 min pre-incubation at 37 °C, NADPH (1 mM) or phosphate buffer was added (final incubation volume of 200 μL) and a 60 min incubation was carried out at 37 °C. Then, samples were quenched with acetone, the precipitated proteins were collected and washed using a Brandel cell harvester, and the presence of radiolabeled material bound to microsomal proteins was measured by scintillation counting after protein solubilization.
29. Compound **3** (10 μM) and 1 mg microsomal proteins/mL in phosphate buffer (pH 7.4) were incubated for 1 h at 37 °C.
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32. Researchers in (OSI) Prosidion reported that they detected a peak having the mass spectra of a thiourea metabolite after administration of their thiazol-2-yl amide derivative.[12]