Interaction of the Electrophilic Ketoprofenyl-Glucuronide and Ketoprofenyl-Coenzyme A Conjugates with Cytosolic Glutathione S-Transferases

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ABSTRACT:

Carboxylic acid-containing drugs are metabolized mainly through the formation of glucuronide and coenzyme A esters. These conjugates have been suspected to be responsible for the toxicity of several nonsteroidal anti-inflammatory drugs because of the reactivity of the electrophilic ester bond. In the present study we investigated the reactivity of ketoprofenyl-acylg glucuronide (KPF-OG) and ketoprofenyl-acetyl-coenzyme A (KPF-SCoA) toward cytosolic rat liver glutathione S-transferases (GST). We observed that KPF-SCoA, but not KPF-OG inhibited the conjugation of 1-chloro-2,4-dinitrobenzene and 4-nitroquinoline N-oxide catalyzed by both purified cytosolic rat liver GST and GST from FAO and H5-6 rat hepatoma cell lines. Photoaffinity labeling with KPF-SCoA suggested that the binding of this metabolite may overlap the binding site of 4-methylumbelliferone sulfate. Furthermore, high-performance liquid chromatography and mass spectrometry analysis showed that both hydrolysis and transacylation reactions were observed in the presence of GST and glutathione. The formation of ketoprofenyl-S-acyl-glutathione could be kinetically characterized (apparent \( K_m \) = 196.0 \( \pm \) 70.8 \( \mu \)M). It is concluded that KPF-SCoA is both a GST inhibitor and a substrate of a GST-dependent transacylation reaction. The reactivity and inhibitory potency of thioester CoA derivatives toward GST may have potential implications on the reported in vivo toxicity of some carboxylic acid-containing drugs.

The development of pharmacovigilance tools over the years has led to the detection of an increasing number of reports of adverse effects of drugs (reviewed by Lee, 2003). Among those, the proportion of carboxylic acid drugs withdrawn from the market has been strikingly elevated. Carboxylic acid-containing nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in medicine because of their analgesic, antipyretic, and anti-inflammatory activities. Most frequently, the adverse drug reactions resulted in injury to the liver, which is central for the metabolism of xenobiotics. Consequently, it has been proposed that protein-drug adducts resulting from the metabolism of some of these carboxylic acid substances may be associated with the incidence of adverse drug reactions occasionally observed (Boelsterli, 2002). 2-Aryl propionic acid-containing NSAIDs are metabolized by the phase II detoxification enzymes, UDP-glucuronosyltransferases. Although this mechanism is often considered as a detoxifying system, a few examples of bioactivation through glucuronidation have been described in the case of carboxylic acid-containing drugs, leading to the formation of electrophilic acylglucuronides (Ritter, 2000). In addition to glucuronidation, xenobiotics bearing a carboxylic acid group can undergo a conjugation reaction to coenzyme A, catalyzed by both mitochondrial and microsomal acyl-CoA synthetases, and resulting in the formation of acyl-CoA thioesters of the corresponding compounds. This is, for instance, the case of the 2-arylpropionic acid drug ketoprofen (KPF), which is both glucuronidated (Sabolovic et al., 2004; Sakaguchi et al., 2004) and thio-esterified with CoA (Carabaza et al., 1996). The latter metabolite appears to be an obligatory

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; KPF, ketoprofen (2-(3-benzoylphenyl) propionic acid); COX, cyclooxygenase; KPF-OG, ketoprofenyl-acylg glucuronide; KPF-SCoA, ketoprofenyl-acetyl-coenzyme A; GST, glutathione S-transferases; GSH, glutathione reduced form; CDNB, 1-chloro-2,4-dinitrobenzene; NQO, 4-nitroquinoline N-oxide; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; PSD, post source decay; MS, mass spectrometry; KPF-SG, ketoprofenyl-S-acyl-glutathione; 4-MU sulfate, 4-methylumbelliferone sulfate.
advantage of the photoactivatable properties of the benzophenone
efficient substrate of GST Mu and Pi (Aceto et al., 1990). Taking
4-nitroquinoline
Theta and therefore is considered as a diagnostic GST substrate, and
zene (CDNB), which is catalyzed by all cytosolic GST except GST
evaluated on the conjugation of GSH to both 1-chloro-2,4-dinitroben-
kinetically characterized in the present work. These effects were
of KPF-SCoA in the presence of these enzymes was elucidated and
SCoA toward cytosolic rat liver GST was evaluated and the reactivity
esters of xenobiotics can react with reduced glutathione (GSH), the
inhibitors of recombinant GST (Silva et al., 1999), and 3) several CoA
metabolites was dictated by previous reports showing that 1) various
electrophilic metabolites. A study of the interaction of KPF-OG and KPF-SCoA with cytosolic GST was
undertaken. The choice of these enzymes as putative targets for these
KPF acyl derivatives are chemically reactive species that can
trigger the formation of adducts with various proteins such as albumin
(Presle et al., 1996), UDP-glucuronosyltransferases (Terrier et al.,
1999), the cyclooxygenase COX-2 (Levoin et al., 2004), and glucose-
6-phosphate dehydrogenase (Asensio et al., 2007).
In the present study we used the main metabolites of KPF, namely
ketoprofen-acyl-glucuronide (KPF-OG) and ketoprofen-acyl-coenzyme A (KPF-SCoA), as acyl metabolite model compounds from
carboxylic acid-containing NSAIDs (Fig. 1) to gain further insights into the reactivity of these electrophilic metabolites. A study of the
interactions of KPF-OG and KPF-SCoA with cytosolic GST was undertaken. The choice of these enzymes as putative targets for these
metabolites was dictated by previous reports showing that 1) various
electrophilic esters can be substrates for GST (Hayes et al., 2005),
including acylglucuronides (Shore et al., 1995) and acyl-CoA deriv-
avatives (Grillo and Benet, 2002), 2) fatty acyl-CoA esters are potent
inhibitors of recombinant GST (Silva et al., 1999), and 3) several CoA
esters of xenobiotics can react with reduced glutathione (GSH), the
cosubstrate of GST, leading to the corresponding thioester derivatives (references therein). The inhibition potency of KPF-OG and KPF-
SCoA toward cytosolic rat liver GST was evaluated and the reactivity of KPF-SCoA in the presence of these enzymes was elucidated and
kinetically characterized in the present work. These effects were evaluated on the conjugation of GSH to both 1-chloro-2,4-dinitroben-
(CDNB), which is catalyzed by all cytosolic GST except GST
Theta and therefore is considered as a diagnostic GST substrate, and
4-nitroquinoline N-oxide (NQO), which has been reported to be an
efficient substrate of GST Mu and Pi (Aceto et al., 1990). Taking
advantage of the photoactivatable properties of the benzophenone
moiety, the two metabolites of KPF were used as photoaffinity labels to study their interaction with GST.

Materials and Methods

Materials. GSH was purchased from Acros (Noisy-le-Grand, France). Purified GST from rat liver, (RS)-ketoprofen [R,S-2-(3-benzoylphenyl) propi-
onic acid], S-ketoprofen, and all other chemicals were purchased from Sigma-
Aldrich (St. Quentin Fallavier, France) and were of the highest available
degree of purity. Cell culture reagents were from Eurobio (Courtaboeuf,
France). KPF-OG was prepared using a previously published enzymatic
method (Terrier et al., 1999). The chemical synthesis of KPF-SCoA has been
described previously (Levoin et al., 2002). Synthesis of ketoprofenyl-S-acyl-
glutathione was performed as described below. The rat hepatoma Fao and H5-6
cell lines have been cloned from the H4IIEC3 cell line (Pitot et al., 1964)
derived from Reuber H35 hepatoma cells (Reuber, 1961).

Instrumentation. HPLC was performed on a Waters system equipped with a Waters 510 pump and a Waters 996 photodiode array detector at 254 nm
(Waters, Milford, MA). MALDI-TOF experiments were performed in positive ion mode and post source decay (PSD) with a Bruker Reflex IV instrument
(Bruker-Franzen Analytik GmbH, Bremen, Germany). For the MALDI-TOF MS sample preparation, 1 µl of 2,5-dihydroxybenzoic acid matrix solution (0.1
M in equal volumes of water-acetonitrile) was added to 1 µl of the sample,
placed on the sample plate and allowed to dry in an air stream. Ionization was
achieved by using a nitrogen laser (λ = 337 nm, pulse duration = 4 ns, output
g = 400 µJ, repetition rate = 5 Hz). A reflectron mode with a total acceleration voltage of 20 kV and an extraction delay time of 200 ns was used
for the mass spectrometry analysis. The ion assignment was attained after external calibration performed with polyethylene glycol 400 Na+ and K+ cationized ions.

Chemical Synthesis of Ketoprofenyl-S-Acyl-Glutathione. Ketoprofenyl-
S-acyl-glutathione (KPF-SG) was chemically synthesized using a method
modified from the synthesis of acyl-CoA-ketoprofen conjugates (Levoin et al.,
2002), by substituting GSH for CoASH. To a solution of KPF (61 mg, 0.24
mmol) in 5.5 ml of anhydrous dichloromethane was added a solution of
2,6-lutidine (28 mmol) in 5.5 ml of anhydrous dichloromethane. The mixture was stirred for 1 h at
room temperature under a nitrogen atmosphere. The formation of the activation
KPF reaction intermediate was monitored by HPLC (retention time 6.4 min) as described (Levoin et al., 2002). When the reaction was completed, the mixture was concentrated to dryness and solubilized in tetrahydrofuran (4.5 ml). GSH (46.2 mg, 0.15 mmol in 4.5 ml of distilled water) was then added, the pH adjusted to 6.2 with NaOH (5 N), and the mixture was stirred at room temperature for 2 h under a nitrogen atmosphere. The formation of KPF-SG was followed by HPLC as described below. Finally, tetrahydrofuran was evaporated, and the aqueous layer was extracted with hexane to remove unreacted products and concentrated. KPF-SG from the aqueous phase was purified by semipreparative HPLC as described below. The residue was characterized by MALDI-TOF positive ion mass spectrum (see Fig. 5).

Inhibition of Purified Rat Liver GST Activities. GST activities were determined using CDNB as substrate, essentially as described by Habig et al. (1974). The enzymatic activity of purified rat liver cytosolic GST (1.23 µg of protein/ml) was assayed at 340 nm (Uvikon 941 spectrophotometer; Kontron Instruments, SECOMAM, Alès, France) in 0.1 M sodium phosphate buffer (pH 6.5) at 30°C for 4 min using GSH (3 mM in 0.1 M sodium phosphate buffer, pH 6.5) and CDNB (4 mM in dimethyl sulfoxide). When 4-NQO (0.1 mM in dimethyl sulfoxide) was used as the acceptor substrate, the assay was performed as described by Stanley and Benson (1988), except that GSH concentration was held at 2 mM (0.1 M sodium phosphate buffer, pH 6.5). For inhibition experiments, the tested substances (0.1 mM) were dissolved in Milli-Q water (CoASH, KPF-OG, and KPF-SCoA) or in dimethyl sulfoxide (KPF and 4-methylumbelliferone sulfate) and included in the GST assays. Solvents were added at 2% (v/v) in the assays and did not affect enzyme activities (results not shown). The residual activity (as a percentage) in the presence of the inhibitors was calculated, with control (2% solvent) representing 100% activity.

Inhibition of GST in Cell Homogenates. The rat hepatoma cell lines FAO and H5-6 were grown to subconfluency in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. These cells were cultured in 250-ml Falcon flasks (BD Biosciences, Bedford, MA) at 37°C in a humidified atmosphere containing 5% CO2. Cells were scraped into phosphate-buffered saline and centrifuged (4°C, 500g) for 5 min. The pellet was resuspended in 0.1 M ice-cold sodium phosphate buffer (pH 7.0), sonicated twice for 10 s (VibraCell; Fisher Bioblock Scientific, Illkirch, France), and centrifuged (4°C, 2500g) for 15 min. The supernatant was kept at −80°C until the GST assay. The inhibitory potency of KPF, KPF-SCoA, and KPF-OG (0.1 mM) on GST activities (CDNB and 4-NQO) in the FAO and H5-6 cell homogenates was evaluated as described for purified rat liver GST but with final protein concentrations of 0.153 and 0.468 mg/ml (for initial rate conditions), respectively, in the assay.

Photoaffinity Labeling of Purified Rat Liver GST with Ketoprofenyl-CoA. Purified cytosolic rat liver GST (0.16 mg/ml) were placed into 1.5-ml microtubes and incubated for 0 to 2 h at 4°C under a 365-nm UV lamp (Spectroline; Polylabo, Strasbourg, France) in 0.1 M sodium phosphate buffer (pH 7.4) and in the presence of KPF-SCoA (0.5 mM). UV-irradiated samples were withdrawn at different irradiation times (0–2 h) and diluted 100 times in 0.1 M sodium phosphate buffer (pH 6.5) before determination of GST activity using CDNB as the acceptor substrate as described above. The effect of UV irradiation alone was evaluated by performing the experiment in parallel in the absence of KPF-SCoA. The residual activity (as a percentage) was calculated from the enzyme activity in the nonirradiated assay at t0 representing 100% residual activity. GST activity protection against photoactivation by KPF-SCoA (0.25 mM) was studied in the presence of GSH (4 mM in 0.1 M sodium phosphate buffer, pH 6.5), CDNB (4 mM), 4-MU sulfate (2 mM), estrone 3-sulfate (2 mM), and the corresponding solvent alone (1% v/v), using the experimental procedures described above and an irradiation time of 2 h.

Protein Assays. Protein concentrations of cell homogenates and purified GST were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

HPLC Analysis. Reaction products resulting from the incubation of GST and/or GST with KPF-SCoA were analyzed by reversed-phase HPLC with UV detection at 254 nm. Samples (10 µl) were injected in a LiChrophen RP-18 column (18.5 µm, 125 × 4 mm; Merck KGaA, Darmstadt, Germany). The mobile phase consisted of 45% 9 mM sodium phosphate buffer (pH 5.5) and 55% methanol, and isocratic elution was carried out at a flow rate of 1.5 ml/min.

Reactivity of KPF-SCoA with GSH in the Presence of GST. GSH (3 mM in 0.1 M sodium phosphate buffer, pH 6.5) was incubated at 37°C in 0.1 M sodium phosphate buffer (pH 6.5) in presence of KPF-SCoA (0.5 mM) and with or without GST (2.34 mg of proteins/ml in 0.1 M sodium phosphate buffer, pH 6.5). A sample (23.44 µg of proteins) was withdrawn from the mixture as a function of time (0–4 h) and was mixed with 4 volumes of methanol to stop the reaction and precipitate the proteins. After centrifugation (5 min, 12,000g, 4°C) supernatants were stored at −80°C until HPLC analysis. Controls were also run in parallel under the same conditions but with KPF-SCoA or CoASH alone (0.5 mM), glutathione disulfide (3 mM) in place of GSH, and heat-denatured GST, respectively. The KPF-SCoA-concentration dependence of the reaction catalyzed by GST was studied by incubating GST (2.34 mg of proteins/ml) and GSH (3 mM) with KPF-SCoA (0.01–1 mM) in 0.1 M sodium phosphate buffer (pH 6.5) at 30°C during 45 min. The reaction mixtures were then treated and analyzed by reversed-phase HPLC as described above. Controls without GST were run in parallel to account for the uncatalyzed reaction.

Quantification of Ketoprofenyl-S-Acyl-Glutathione. KPF-SCoA produced in the presence of GST was quantified as follows. KPF-SG fractions eluted from the HPLC column were collected and fully hydrolyzed in the presence of an equal volume of NaOH (0.5 N) for 2 h at 40°C. The amount of KPF-SG was deduced from the amount of KPF released, the latter being quantified using a KPF standard curve and HPLC separation.

Statistical Analysis. Values are expressed as the mean in residual activities ± S.D. of at least two experiments performed in triplicate.

Results

Inhibition of GST by Ketoprofenyl-SCoA and Ketoprofenyl-Glucuronides. The effect of the two main acyl metabolites of ketoprofen was first evaluated on purified rat liver cytosolic GST to find out whether GST are possible targets of these compounds (Fig. 2A). KPF is a chiral NSAID that has been reported to inhibit GST (Sadzuka et al., 1994). It was tested in the present study either as racemates (as administered) or as the S-enantiomer (the form with anti-inflammatory properties due to the potent inhibition of COX). KPF was found to more selectively inhibit the conjugation of 4-NQO, an efficient substrate of GST Mu and Pi. S-KPF inhibition was similar to that observed with the KPF racemic mixture (results not shown). The inhibitory potency of KPF was lost once it was conjugated to glucuronic acid (Fig. 2A). If CoASH alone did not significantly affect the GST activity, KPF-SCoA could inhibit these enzymes (Fig. 2A). Likewise, cytosolic GST activities from the FAO and H5-6 rat hepatocarcinoma cell lines were significantly inhibited by KPF-SCoA, but not by the acylglucuronide (Fig. 2B).

We next characterized the mode of inhibition of GST by KPF-SCoA. A Lineweaver-Burk representation could not be shown because of the relatively low amount of this metabolite available for this study. Therefore, another approach was undertaken, using KPF-SCoA as a photoaffinity probe combined with ligand protection experiments. It was indeed reported previously that KPF can bind to proteins upon UV irradiation through the benzophenone moiety (Boscá et al., 1994; Chuang et al., 1999). We observed in preliminary experiments that KPF could photolabel GST (E. Battaglia and D. Bagrel, unpublished observations). Furthermore, irradiation of purified rat liver GST in the presence of 0.5 mM KPF-SCoA led to a time-dependent inactivation, reaching 72% inactivation after 2 h (Fig. 3A). Conversely, no inhibition of the conjugation reaction could be detected in the absence of irradiation in these experimental conditions. No significant protection was observed when the labeling was performed in the presence of either GSH, CDNB (Fig. 3B), or 2 mM estrone 3-sulfate (not shown), whereas preincubation of the enzymes with 2 mM 4-MU sulfate before labeling with 0.25 mM KPF-SCoA brought almost full pro-
tection against photoinactivation. KPF-OG photolabeling of GST under similar conditions did not trigger any significant enzyme inactivation (data not shown). This suggests that KPF-SCoA binds within the 4-MU sulfate binding site.

**KPF-SCoA Reactivity in the Presence of GST.** Because KPF-SCoA (but not KPF-OG) exerts an inhibitory effect toward GST, we next determined the possible reactivity of the former metabolite with GSH and evaluated the possibility of a reaction mediated by these enzymes. A representative reversed-phase HPLC chromatogram obtained after incubation of KPF-SCoA with GSH at pH 6.5 in the presence of purified rat liver GST is shown in Fig. 4. Several species with different retention times were detected at 254 nm. The retention times of ~2.8 and 3.3 min matched those of authentic KPF-SCoA R- and S-enantiomers and therefore were considered as unreacted metabolites. This assignment was confirmed by mass spectrometric analysis with MALDI-TOF positive ion mass spectrum in accordance with the structures of the acyl-CoA metabolites (data not shown). The compound eluted at ~5.4 min comigrated with authentic KPF, and the detection of the protonated molecular ion MH⁺ m/z 255.33 is consistent with the structure of KPF (data not shown). The structure of the compound eluted at ~4.7 min could not be proposed on the basis of the retention time from the HPLC analysis and was therefore further characterized by mass spectrometry.

**MS Identification of 2-(3-Benzoylphenyl) Propionic Acid-Glutathione.** A transacylation product resulting from the reaction be-

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**Fig. 2.** Inhibitory effect of KPF and its acyl metabolites toward GST activities of purified enzyme and rat hepatoma cell homogenates. A, the inhibition of the activity of purified rat liver cytosolic GST was evaluated in the presence of the indicated compounds (0.1 mM) using either CDNB [□] or 4-NQO [□] as substrate, under initial rate conditions. B, the inhibitory effect of KPF, KPF-SCoA, and KPF-OG (0.1 mM) toward GST was evaluated using 4-NQO as substrate on FAO [□] and H5-6 [□] rat hepatoma cells homogenates. Residual activity (as a percentage) was expressed as the ratio between the activity in the presence of the compounds and in the absence of compound (solvent alone) × 100. *, p < 0.05 versus control (solvent alone) (n = 3).

**Fig. 3.** Photoaffinity labeling of purified rat liver GST by KPF-SCoA. A, time-dependent GST photoinactivation in the presence of KPF-SCoA. Rat liver GST (0.16 mg/ml) was incubated in the presence of KPF-SCoA (0.5 mM) and irradiated [●] or not [●] at 365 nm for 0 to 120 min. The GST was also irradiated in the absence of KPF-SCoA [●]. The mixture was then diluted 100 times in 0.1 M sodium phosphate buffer (pH 6.5), and residual activity was calculated as described in Fig. 2. B, ligand protection against GST inactivation by KPF-SCoA. The irradiation of rat liver GST was performed in the presence KPF-SCoA as described in A but for a single time point (120 min). GST was irradiated either in the presence of 0.25 mM KPF-SCoA alone or combined with 2 mM of the GST ligands 4-MU sulfate, GSH, or CDNB. The mixture was subsequently diluted 100 times, and residual activity was calculated as described in the legend to Fig. 2, using the enzyme activity found in the absence of irradiation as corresponding to 100% activity (n = 3). *, p < 0.05.
between GSH and KPF-SCoA was postulated on the basis of the electrophilic properties of the thioester bound, as well as previous reports on similar metabolites (Shore et al., 1995; Grillo and Benet, 2002; Li et al., 2002, 2003a; Olsen et al., 2002; Grillo et al., 2003). Mass spectrometric analysis was performed to allow the identification of the reaction products separated by reversed-phase HPLC. Furthermore, authentic 2-(3-benzoylphenyl) propionic acid-glutathione (KPF-SG) resulting from such postulated transacylation was chemically synthesized by coupling ketoprofen to GSH using the mixed anhydride method (Levoin et al., 2004) and compared with the enzymatic reaction products resulting from the incubation of GST with KPF-SCoA and GSH. MALDI-TOF mass spectrometry analysis of KPF-SG obtained from chemical and enzymatic synthesis is shown in Fig. 5. A characteristic protonated molecular ion MH$^+$/m/z 544 and the potassium adduct ion m/z 582 were detected for the authentic KPF-SG resulting from the chemical synthesis, whereas the potassium adduct ion m/z 582 was not found in the enzymatic reaction products (Fig. 5, A and B). The lack of the potassium adduct may be due to the weak concentration of potassium in the solution analyzed. Likewise, analysis by the PSD technique provided a mass spectrum of the product KPF-SG obtained by the chemical method that was identical to that acquired during the mass spectrometric analysis of the product obtained by the enzymatic method. The PSD spectrum of KPF-SG MH$^+$ ion m/z 544 showed characteristic fragment ions m/z 500, 469, 415, 308, 274, and 237 (Fig. 5C) originating from the proposed cleavages depicted in this figure.

Kinetic Studies of the Hydrolytic and Transacylation Reactions Catalyzed by GST. We characterized the GST-mediated transacylation and KPF-SCoA hydrolysis by evaluating the time dependence and saturation pattern of these reactions. The coincubation of KPF-SCoA and GSH resulted in a significant uncatalyzed reaction that varied from 3.5 to 13.9% of the reaction observed in the presence of GST as a function of the incubation time (Fig. 6A). Therefore, the sole contribution of the enzyme-catalyzed reaction was evaluated by deducing from the amount of the total reaction products that measured in the absence of enzyme (Fig. 6A). Incubation of KPF-SCoA with GSH and GST resulted in a time-dependent formation of KPF, the later being linear for up to 4 h (Fig. 6A), defining within these experimental conditions the time frame of the initial rate for the hydrolytic reaction. Moreover, the kinetics of KPF-SG formation was linear for up to 1 h. Accordingly, an incubation time of 45 min was chosen to evaluate the substrate saturation pattern of the enzymatic reactions (hydrolysis and transacylation). A saturation curve was observed for KPF-SCoA concentrations ranging from 0.01 to 1 mM (Fig. 6B). A double reciprocal plot allowed the determination of an apparent $K_m$ toward KPF-SCoA of 196.0 ± 70.6 μM for the transacylation reaction (Fig. 6C). On the other hand, the hydrolysis reaction resulting in the formation of KPF and CoASH from KPF-SCoA was too slow to allow the determination of kinetic parameters for this reaction (data not shown). Because proteins contain multiple nucleophilic amino acid residues, such as cysteiny1 residues that could account for the observed reactivity of KPF-SCoA (independently of the enzyme integrity), we evaluated the hydrolytic and transacylation reactions after thermal denaturation of the purified GST. The products were detected by HPLC at levels similar to that found with the uncatalyzed reaction (data not shown). Furthermore, the free thiol moiety of GSH appears to be essential for the reaction as no products could be detected when glutathione disulfide was substituted for GSH (data not shown).

**Discussion**

A number of carboxylic acid drugs have been withdrawn from the market after observation of unacceptable levels of hepatotoxicity, and these idiosyncratic drug reactions have been hypothesized to be associated to the formation of chemically reactive substances. Thus, various acylglucuronides have been shown to be electrophilic metabolites that can spontaneously react with proteins both in vitro and in vivo, leading to adduct formation (Sallustio et al., 2000). This reactivity is not limited to proteins because electrophilic acylglucuronide conjugates have been reported to react with the tripeptide glutathione,
the cosubstrate for the conjugation reaction carried out by GST (Shore et al., 1995; Grillo and Hua, 2003). A second bioactivation pathway involves the formation of electrophilic acyl-CoA derivatives by acyl-CoA synthetases. Acyl-CoA derivatives of xenobiotics can react with proteins and GSH, resulting in adduct formation through nucleophilic acyl substitution (Sidenius et al., 2004).

KPF has been previously reported to inhibit GST (Sadzuka et al., 1994). In the present study, we characterized the inhibitory potency and reactivity toward GST of the two main acylated metabolites of KPF, namely KPF-OG and KPF-SCoA (Fig. 1). Whereas KPF-OG did not affect GST activity, we observed that KPF-SCoA was able to inhibit both CDNB and NQO conjugation to GSH catalyzed by purified rat liver GST (Fig. 2). A similar inhibitory pattern was found in homogenates from H5-6 and FAO rat hepatocarcinoma cells, and the inhibition potency of KPF-SCoA was similar to that observed with KPF (Fig. 2). The lower inhibitory potency observed in the presence of cytosolic extracts than with purified GST is likely to be due to the buffering effect of additional proteins interacting with the two acyl metabolites of KPF. Various proteins such as albumin (Li et al., 2003b), sulfotransferases (Tulik et al., 2002), COX (Levoin et al., 2004), hepatocyte nuclear factor-4 alpha (Hertz et al., 2001), and glucose-6-phosphate dehydrogenase (Asensio et al., 2007) have indeed been reported to interact with CoA thioester derivatives. It was found previously that long-chain saturated fatty acyl-CoAs and peroxisome proliferator-CoA thioesters exert high affinity toward GST (Silva et al., 1999). Our results provide another example of the ability of carboxylic acid-containing compound to bind to GST. We observed that this binding resulted in the inhibition of the conjugation activity of these enzymes.

Taking advantage of the ability of KPF to bind covalently to its protein targets upon UV irradiation (Chuang et al., 1999), we used KPF-SCoA as a photoaffinity label to partially characterize the GST binding site of this metabolite (Fig. 3). A UV-dependent GST inactivation by KPF-SCoA was detected as well as major protection in the presence of 4-MU sulfate, whereas GSH, CDNB, and estrone 3-sulfate did not exert any protective effect. Sulfoconjugates of steroids have been reported to bind in a so-called “ligandin” or “nonsubstrate” site in the GST 1-1 isozyme of rat liver GST (Barycki and Colman, 1997). Conversely, aromatic sulfoconjugates such as benzyl sulfate are GST substrates in the presence of GSH (Gillham, 1971). Interest-
The reactivity of KPF-SCoA as a potential GST substrate has been evaluated. The chemical reactivity of the electrophilic thioester bound for a variety of xenobiotic-SCoA metabolites toward the sulphydryl moiety of cysteine in both low molecular weight GSH and proteins has been reported previously (Shore et al., 1995; Grillo and Benet, 2002; Li et al., 2002, 2003a; Olsen et al., 2002; Grillo et al., 2003). Furthermore, xenobiotic-SCoA metabolites were found to be much more reactive toward GSH than acylglucuronides (Olsen et al., 2002). Whereas Silva et al. (1999) highlighted the absence of metabolism and hydrolysis of the thioester derivatives, interestingly, a transacylation reaction with GSH, enhanced in the presence of GST, was detected in the present study. MALDI-TOF MS analysis of KPF-SG obtained by enzymatic synthesis led to the identification of the expected ion MH+ \( m/z \) 544 (Fig. 5B) in accordance with the proposed structure. This profile was similar to that obtained with chemically synthesized KPF-SG (Fig. 5A). Hence, we concluded that KPF-SCoA was converted to KPF-SG in the presence of GST.

Using the hypolipidemic drug clofibric acid, Grillo and Benet (2002) reported that rat liver GST increase the rate of clofibryl-S-acyl-glutathione formation from a mixture of clofibryl-S-acyl-CoA and GSH, but the kinetic parameters of the catalytic reaction were not determined. In the present study, an apparent \( K_m \) of 196.0 ± 70.6 μM was determined for the transacylation reaction. This value is similar to those for CDNB-conjugating enzymes. GST catalyze miscellaneous GSH-dependent reactions such as nucleophilic aromatic substitution, Michael-type addition, double bond isomerization, and hydroperoxide reduction (reviewed by Mahajan and Atkins, 2005). In addition to the transacylation reaction, the hydrolysis of KPF-SCoA, enhanced in the presence of GST, was detected, but this hydrolysis occurred at a lower rate (Fig. 6A). A relation between the rate of spontaneous transacylation and hydrolysis of CoA thioesters has been reported (Sidenius et al., 2004). The amounts of reaction products (hydrolysis and transacylation) were reduced compared with those observed in the absence of enzyme when GST were heat-inactivated (results not shown), demonstrating that native enzymes contribute to these reactions. The hydrolytic reaction of KPF-SCoA did not result in a saturation pattern, which therefore precluded the determination of the kinetic parameters for this reaction. Hydrolysis of xenobiotic-CoA metabolites has been reported previously, but this reaction was not studied in the presence of enzymes (Li et al., 2003a; Sidenius et al., 2004). Furthermore, the hydrolytic activity of GST on GSH esters (reverse reaction of GSH conjugation) has been demonstrated (Dietze et al., 1998; Ibarra et al., 2003). It is not established, to the best of our knowledge, whether carboxylic acid-SCoA conjugates are directly hydrolyzed by GST or are first transacylated by GSH before hydrolysis by GST (reverse conjugation).

Glutathione thioesters of carboxylic acid-containing drugs may be excreted unchanged in the bile (Shore et al., 1995) or degraded before excretion in urine. Thus, clofibryl-S-acyl-GSH has been shown to be further metabolized in vivo and in vitro to S- to N-acyl-cysteine amide derivatives with the participation of \( \gamma \)-glutamyl transferase (Grillo and Benet, 2001). More recently, the nonsteroidal anti-inflammatory carboxyl drug, zomepirac, was found to be bioactivated to its acyl CoA metabolite, an obligatory intermediate to the in vivo formation of glycine, taurine, and carnitine conjugates (Olsen et al., 2005). A \( \gamma \)-glutamyl transferase-mediated degradation of clofibryl-S-acyl-GSH resulting in the formation of S- and N-acyl-cysteine amide derivatives was detected in rat both in vitro and in vivo (Grillo and Benet, 2001).

Whether xenobiotic acyl-GSH conjugates and their subsequent me-
tabolites may be considered as detoxification products remains to be established.

In conclusion, we report for the first time that, unlike KPF-OG, KPF-SCoA is an inhibitor of both purified and cell homogenate GST. KPF-SCoA is also transacylated in the presence of GSH and hydrolyzed through reactions mediated by purified rat liver GST. The physiotoxicological consequences of GST inhibition, transacylation, and hydrolysis reactions in the presence of CoA thioesters will deserve future investigations.

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References


