Interaction of the electrophilic ketoprofenyl-glucuronide and ketoprofenyl-coenzyme A conjugates with cytosolic glutathione S-transferases

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Abbreviations used: COX, cyclooxygenases, GST, glutathione S-transferases; GSH, glutathione reduced form; GSSG, glutathione disulfide; CDNB, 1-chloro-2,4-dinitrobenzene; NQO, 4-nitroquinoline N-oxide; KPF, ketoprofen (2-(3-benzoylephnyl) propionic acid); KPF-SCoA, ketoprofenyl-acyl-Coenzyme A; KPF-OG, ketoprofenyl-acylglucuronide; KPF-SG, ketoprofenyl-S-acylglutathione; 4MU sulfate, 4-methylumbelliferone sulfate; NSAIDs, nonsteroidal anti-inflammatory drugs.
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. We investigated in the present study the reactivity of ketoprofenyl-acylglucuronide (KPF-OG) and ketoprofenyl-acyl-Coenzyme A (KPF-SCoA) towards 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 KPF-S-acyl-glutathione could be kinetically characterized (apparent $K_M = 196.0 \pm 70.6 \mu M$). 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 towards 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 Among those, the proportion of carboxylic acid drugs withdrawn from the market is strikingly elevated. Carboxylic acid-containing non steroidal anti-inflammatory drugs (NSAIDs) are widely used in medicine due to 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 to 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
The latter metabolite appears to be an obligatory intermediate in the epimerization of chiral profen drugs, such as KPF, as well as in the formation of glycine, taurine and carnitine conjugates (Olsen et al., 2005). KPF, among other drugs, has been used as a model compound to study the possible reactivity of acyl metabolites with cellular proteins. Thus, it was previously observed that both of these KPF acyl derivatives are chemically reactive species which 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).

We have used in the present study the main metabolites of KPF, namely ketoprofenyl-acylglucuronides (KPF-OG) and ketoprofenyl-acyl-coenzyme A (KPF-SCoA) as acyl metabolites model compounds from carboxylic acid-containing NSAIDs (Fig.1), to gain further insights into the reactivity of these electrophilic metabolites. A study of the interaction of KPF-OG and KPF-SCoA with cytosolic GST was here undertaken. The choice of these enzymes as putative target for these metabolites was dictated by previous reports showing that (1) various electrophilic esters can be substrates for GST (Hayes et al., 2005), including acyl glucuronides (Shore et al., 1995) and acyl CoA derivatives (Grillo and Benet, 2002), (2) fatty acyl coA 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 towards 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 CDNB which is catalyzed by all cytosolic
GST except GST Theta and therefore is considered as a diagnostic GST
substrate, and 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 \([R,S-2-(3-benzoylphenyl)\) propionic acid], \(S\)-Ketoprofen, and all others 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 previously described (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 (Milford, MA) equipped with a Waters 510 pump and a Waters 996 photodiode array detector at 254 nm. 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 \(\mu\)L of 2,5-dihydroxybenzoic acid matrix solution (0.1 M in equal volumes of water/acetonitrile) was added to 1 \(\mu\)L of the sample, placed on the sample plate and allowed to dry in an air stream. Ionization was achieved by using a nitrogen laser \((\lambda = 337\) nm, pulse duration = 4 ns, output energy = 400 \(\mu\)J, 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 anhydrous dichloromethane was added a solution of 2,6 lutidine (28 µL, 0.24 mmol) and ethyl chloroformate (23 µL, 0.24 µmol in 1.85 mL anydrous dichloromethane). The mixture was stirred for 1 hour at room temperature under nitrogen atmosphere. The formation of the activated 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 mmole in 4.5 mL distilled water) was then added, the pH adjusted to 6.2 with NaOH (5N), and the mixture stirred at room temperature for 2 hours under nitrogen atmosphere. The formation of KPF-SG was followed by HPLC as described below. Finally, tetrahydrofuran was evaporated and the aqueous layer extracted with hexane to remove unreacted products and concentrated. KPF-SG from the aqueous phase was purified by semi-preparative 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 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, essentially as described by (Habig et al., 1974). The enzymatic activity of purified rat liver cytosolic GST (1.23 µg proteins/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 DMSO). When 4-nitroquinoline N-oxide (4NQO, 0.1 mM in dimethylsulfoxide) 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 milliQ® water (CoASH, KPF-OG and KPF-SCoA) or in dimethylsulfoxide (KPF, 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 (%) 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 DMEM 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 in Phosphate Buffered
Saline and centrifuged (4°C, 500 g) for 5 min. The pellet was resuspended in 0.1 M ice-cold sodium phosphate buffer (pH 7.0), sonicated twice for 10 seconds (VibraCell, Fisher Bioblock Scientific, Illkirch, France) and centrifuged (4°C, 2500 g) for 15 min. The supernatant was kept at –80°C until GST assay. The inhibitory potency of KPF, KPF-SCoA and KPF-OG (0.1 mM) on GST activities (CDNB and 4NQO) in the FAO and H5-6 cell homogenates was evaluated as described for purified rat liver GST, but with a final protein concentration of 0.153 mg/mL 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 0-2 hours 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) prior to determination of GST activity using CDNB as acceptor substrate, as described above. The effect of UV irradiation alone was evaluated by performing in parallel the experiment in the absence of KPF-SCoA. The residual activity (%) was calculated from the enzyme activity in the non-irradiated assay at t₀ representing 100 % residual activity. GST activity protection against photoinactivation 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), 4MU 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 2h.

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

**HPLC analysis.** Reaction products resulting from the incubation of GST and/or GSH with KPF-SCoA were analyzed by reverse-phase HPLC with UV detection at 254 nm. 10 µL of samples were injected in a Lichrospher RP-18 column (18.5 µm, 125 x 4 mm, Merck KGaA, Darmstadt, Germany). The mobile phase consisted of 45 % of 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 30°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 proteins/mL in 0.1 M sodium phosphate buffer, pH 6.5). A sample (23.44 µg proteins) was withdrawn from the mixture as a function of time (0-4 h) and was mixed to four volumes of methanol to stop the reaction and precipitate the proteins. After centrifugation (5 minutes, 12000g, 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), GSSG (3 mM) in place of GSH, and heat-denaturated GST, respectively.
The KPF-SCoA-concentration dependence of the reaction catalyzed by GST was studied by incubating GST (2.34 mg proteins/mL) and GSH (3 mM) with KPF-SCoA (0.01 mM to 1 mM) in 0.1 M sodium phosphate buffer (pH 6.5) at 30°C during 45 minutes. The reaction mixtures were then treated and analyzed by reverse-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-SG 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 ± standard deviations (S.D.) of at least two experiments performed, at least, in triplicates.
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 if GST are possible targets of these compounds (Fig. 2A). KPF is a chiral NSAID which 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 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 activity 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 performed 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 previously reported that KPF can bind to proteins upon UV-irradiation through the benzophenone
moiety (Bosca et al., 1994; Chuang et al., 1999). We observed in preliminary experiments that KPF could photolabel GST (unpublished observation). 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 two hours (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 carried out in the presence of either GSH, CDNB (Fig. 3B) or 2 mM estrone 3-sulfate (not shown), while preincubation of the enzymes with 2 mM 4MU sulfate prior to labeling with 0.25 mM KPF-SCoA brought near full protection 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 4MU sulfate binding site.

**KPF-SCoA reactivity in the presence of GST.** Since KPF-SCoA (but not KPF-OG) exerts an inhibitory effect towards 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 reverse phase HPLC chromatogram obtained after incubation of KPF-SCoA with GSH at pH 6.5 in the presence 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 min 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 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; Olsen et al., 2002; Grillo et al., 2003; Li et al., 2003a). 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 to 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 are shown in Fig. 5. A characteristic protonated molecular ion MH$^+$ m/z 544 and the potassium adduct ion m/z 582 was detected for the authentic KPF-SG resulting from the chemical synthesis while the potassium adduct ion m/z 582 was not
found in the enzymatic reaction products (Fig. 5A and 5B). The lack of the potassium adduct may be due to the weak concentration of potassium in the analyzed solution. Likewise, analysis by Post Source Decay (PSD) technique provided a mass spectrum of the product KPF-SG obtained by chemical method 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 co-incubation of KPF-SCoA and GSH resulted in a significant uncatalyzed reaction which 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 4h (Fig. 6A), defining within these experimental conditions the time frame of initial rate for the hydrolytic reaction. Moreover, the kinetics of KPF-SG formation was linear for up to 1 h. An incubation time of 45 min was accordingly 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 mM to 1mM (Fig. 6B). A double reciprocal plot allowed the determination of an apparent $K_M$ towards KPF-SCoA of $196.0 \pm 70.6 \mu$M 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). Since proteins contain multiple nucleophilic amino acid residues, such as cysteinyI residues which 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 since 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 acyl glucuronides have been shown to be electrophilic metabolites which can spontaneously react with proteins both \textit{in vitro} and \textit{in vivo} leading to adduct formation (Sallustio et al., 2000). This reactivity is not limited to proteins since electrophilic acyl glucuronide conjugates have been reported to react with the tripeptide glutathione, the cosubstrate for 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 towards GST of the two main acylated metabolites of KPF, namely KPF-OG and KPF-SCoA (Fig. 1). While 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 the one observed with KPF (Fig.
2). The lower inhibitory potency observed in the presence of cytosolic extracts compared to 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-4α (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 previously found that long-chain saturated fatty acyl-CoAs and peroxisome proliferator–CoA thioesters exert high affinity towards GST (Silva et al., 1999). Our results provide another example of carboxylic acid–containing compound able 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), KPF-SCoA was used as a photoaffinity label to partially characterize the GST binding site of this metabolite (Fig. 3). An UV-dependent GST inactivation by KPF-SCoA was detected as well as a major protection in the presence of 4MU sulfate, while 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 alpha-class rat liver GST isozyme 1-1 (Barycki and Colman, 1997). Conversely, aromatic sulfoconjugates such as benzyl sulfate are GST substrates in the presence of GSH (Gillham, 1971). Interestingly, estrone 3-sulfate was also tested in this same work and was found not to be a
GST substrate. Therefore, our experiment using KPF-SCoA as photolabel suggests that it may bind within the electrophilic substrate site (H-site). This is further supported by the fact that KPF-SCoA behaves as a GST substrate as shown by the detection of a GST-dependent transacylation reaction with GSH (Fig. 5 and 6). The observation that the diagnostic GST substrate CDNB does not protect against inactivation even though KPF-SCoA is itself a GST substrate can be explained in view of some previous works. For instance, it has been reported that ethacrynic acid, both a GST inhibitor and a GST substrate, can bind to human GST P1-1 in a nonproductive and productive mode, which suggests the existence of multiple binding sites and binding modes for GST ligands (Oakley et al., 1997). KPF-SCoA binding mode and binding stoichiometry may thus be distinct from that of CDNB.

The reactivity of KPF-SCoA as potential GST substrate has been evaluated. The chemical reactivity of the electrophilic thioester bound for a variety of xenobiotic-SCoA metabolites towards the sulphydryl moiety of cysteine in both low molecular weight GSH and proteins has been indeed previously reported (Shore et al., 1995; Grillo and Benet, 2002; Li et al., 2002; Olsen et al., 2002; Grillo et al., 2003; Li et al., 2003a). Furthermore, xenobiotic–SCoA were found to be much more reactive towards GSH compared to acyl glucuronides (Olsen et al., 2002). While (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 ions MH\(^+\) m/z 544 (Fig. 5B) in accordance with the proposed structure. This profile was similar to that obtained with KPF-SG chemically synthesized (Fig. 5A). Hence we concluded that KPF-CoA was converted to KPF-SG in the presence of GST.

Using the hypolipidemic drug clofibric acid, Grillo and Benet (2002) have 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 \(K_M = 196.0 \pm 70.6 \, \mu M\) was determined for the transacylation reaction. This value is similar to those of CDNB-conjugating enzymes. GST catalyze miscellaneous GSH–dependent reactions such as nucleophilic aromatic substitution, Michael-type addition, double bond isomerisation, 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) was reduced to 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 previously
reported 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 prior to 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 prior to 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 contribution of γ-glutamyltransferase (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 γ-glutamyl transferase-mediated degradation of clofibryl-S-acyl-GSH resulting in the formation of S- and N-acyl-cysteine amide derivatives were detected in rat both in vitro and in vivo (Grillo and Benet, 2001). Whether xenobiotic acyl-GSH conjugates and their subsequent metabolites may be considered as a 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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Footnote
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Legends for figures

Figure 1. Structure of Ketoprofen, Ketoprofen-acylglucuronide and Ketoprofen-acylCoenzyme A. * Chiral center.

Figure 2. Inhibitory effect of KPF and its acyl metabolites towards 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 4NQO (□) as substrate, under initial rate conditions. B. The inhibitory effect of KPF, KPF-SCoA and KPF-OG (0.1 mM) towards GST was evaluated using 4NQO as substrate on FAO (□) and H5.6 (■) rat hepatoma cells homogenates. Residual activity (%) was expressed as the ratio between the activity in the presence of the compounds and in the absence of compound (solvent alone) x 100. * p<0.05 vs control (solvent alone) (n=3).

Figure 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 0-120 min. The GST was also irradiated in the absence of KPF-SCoA (□). The mixture was then diluted 100
times in sodium phosphate buffer 0.1 M (pH 6.5) and residual activity was calculated as described in Figure 2. \textbf{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 4MU sulfate, GSH, or CDNB. The mixture was subsequently diluted 100 times and residual activity was calculated as described in Fig. 2, using the enzyme activity found in the absence of irradiation as corresponding to 100% activity (n=3). * p<0.05.

\textbf{Figure 4.} Representative HPLC chromatogram of the products generated upon incubation of KPF-SCoA with GSH and GST. GST (2.34 mg/mL) were incubated in 0.1 M sodium phosphate buffer (pH 6.5) for 2 h at 30°C in the presence of KPF-SCoA (0.5 mM) and GSH (3 mM) and analyzed by reverse-phase HPLC as described in Material and Methods.

\textbf{Figure 5.} MALDI TOF positive ion mass spectra of KPF-SG formed from (A) chemical synthesis and (B) enzymatic synthesis. (C) PSD analysis of KPF-SG produced by cytosolic rat liver GST in the presence of KPF-CoA and GSH as described in the legend to Fig. 4.
Figure 6. Characterization of the transacylation and hydrolytic reactions of KPF-SCoA catalyzed by purified rat liver GST. A. Time-course of the transacylation and hydrolytic reactions. KPF-SCoA (0.5 mM) and GSH (3 mM) were incubated with purified rat liver GST and the reaction products were analyzed as a function of time by reverse-phase HPLC as described in Material and Methods. Both GST-mediated transacylation leading to KPF-SG (■) and hydrolysis reaction to KPF (●) are plotted after subtracting the products obtained in the presence of GST to those obtained in the absence of the enzymes. Control reactions performed in the same conditions but without GST are also plotted for transacylation (□) and hydrolysis (○) reactions. B. Representative Michaelis plot of the GST-dependent catalytic reaction leading to the formation of the transacylation product KPF-SG. The experimental conditions were as described in A, but with an incubation time of 45 min, and with KPF-SCoA concentrations ranging from 0.01 to 1 mM. C. Lineweaver-Burk plot of B (n=3).
Figure 1

Ketoprofen

Ketoprofen-acylglucuronide

Ketoprofen-acylCoenzyme A
Figure 2

A

residual activity (%)

Control  KPF  KPF-ScOA  CoASH  KPF-OG

B

residual activity (%)

KPF  KPF-ScOA  KPF-OG
Figure 3

A

B

UV
KPF-SCoA
4MU sulfate
GSH
CDNB
residual activity (%)

Time (minutes)

residual activity (%)
Figure 4

![Elution time (min)](image)

$A_{254\text{ nm}}$
Figure 5

A. Chemical synthesis of KPF-SG

Mr = 543
MH⁺ m/z 544
MK⁺ m/z 582

B. Enzymatic synthesis of KPF-SG

Mr = 543
MH⁺ m/z 544

C. PSD spectrum of MH⁺ m/z 544
Figure 6

A

Products (pmoles)

Time (min)

B

KPF-SG (pmoles/min)

[1/KPF-SCoA] (mM)

C

1/V (pmoles/min)^{-1}

1/KPF-SCoA (mM)^{-1}

r^2 = 0.989