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Inhibition of human CYP2B6 by *N*,*N*′,*N*″-triethylenethiophosphoramide is irreversible and mechanism-based

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Abstract

The chemotherapeutic agent *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA) is frequently used in high-dose chemotherapy regimens including cyclophosphamide. Previous studies demonstrated partial inhibition by thioTEPA of the cytochrome P4502B6 (CYP2B6)-catalyzed 4-hydroxylation of cyclophosphamide, which is required for its bioactivation. The aim of our study was to investigate the detailed mechanism of CYP2B6 inhibition by thioTEPA. Using human liver microsomes and recombinant P450 enzymes we confirmed potent inhibition of CYP2B6 enzyme activity determined with bupropion as substrate. ThioTEPA was found to inhibit CYP2B6 activity in a time- and concentration-dependent manner. The loss of CYP2B6 activity was NADPH-dependent and could not be restored by extensive dialysis. The maximal rates of inactivation (K_{inact}) were 0.16 min^{-1} in human liver microsomes and 0.17 min^{-1} in membrane preparations expressing recombinant CYP2B6. The half-maximal inactivator concentrations (K_I) were 3.8 μM in human liver microsomes and 2.2 μM in recombinant CYP2B6. Inhibition was attenuated by the presence of alternative active site ligands but not by nucleophilic trapping agents or reactive oxygen scavengers, further supporting mechanism-based action. Inactivated CYP2B6 did not lose its ability to form a CO-reduced complex suggesting a modification of the apoprotein, which is common for sulfur-containing compounds. Pharmacokinetic consequences of irreversible inactivation are more complicated than those of reversible inactivation, because the drug's own metabolism can be affected and drug interactions will not only depend on dose but also on duration and frequency of application. These findings contribute to better understanding of drug interactions with thioTEPA.

Keywords: Cytochrome P450; CYP2B6; thioTEPA; Mechanism-based inhibition; Suicide-inhibition; Cyclophoshamide

1. Introduction

N,*N*′,*N*″-Triethylenethiophosphoramide (thioTEPA) is a polyfunctional alkylating agent that has been used in the treatment of breast, ovarian and bladder cancer for almost five decades as well as in high-dose chemotherapy regimens with subsequent bone marrow transplantation [1]. ThioTEPA is mainly metabolized to *N*,*N*′,*N*″-triethylenephosphoramide (TEPA), a pharmacologically active compound, by oxidative desulfuration. In rat, 50–80% of the active metabolite TEPA is generated by CYP2B1 and CYP2C11 [2,3]. It is interesting to note that the human CYP enzymes responsible for metabolism of thioTEPA to TEPA were only recently identified as CYP3A4 and CYP2B6 [4]. TEPA itself and, to a lesser extent, the degradation product *N*,*N*′-diethylene *N*″-2-chloroethylphosphoramide (monochloro-TEPA), contribute to the antineoplastic potential [5]. Alternatively, thioTEPA undergoes extensive phase II metabolism leading to conjugation with glutathione catalyzed by glutathione *S*-transferases A1-1 and P1-1, and this step is believed to contribute to drug-resistance to alkylating agents [6]. In addition, drug–drug interactions have also been suspected to be involved in drug non-response or increased toxicity of chemotherapy [7]. ThioTEPA is commonly applied in combination with cyclophosphamide (CP) and carboplatin using standard chemotherapy protocols [8]. The CTCb regime includes all three drugs which are administered simultaneously as 96-h continuous infusions [9] whereas a consecutive administration of thioTEPA, CP, and carboplatin over a period of 4 days using short-time infusions

Abbreviations: CP, cyclophosphamide; CYP, cytochrome P450; ESI, electrospray ionisation; HPLC, high-performance liquid chromatography; OR, NADPH:cytochrome-P450-oxidoreductase; thioTEPA, *N*,*N*′,*N*″-triethylenethiophosphoramide

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was used in the CTC chemotherapeutic protocol [10]. CP is a cancer chemotherapeutic prodrug which unfolds its alkylating activity only after metabolism to 4-hydroxy-CP. Huitema et al. [11] found significantly reduced plasma-levels of 4-hydroxy-CP in patients using a high-dose chemotherapy regime with thioTEPA when thioTEPA was administered prior to CP. Moreover, investigations in human liver microsomes provided evidence for a reduced conversion of CP to 4-hydroxy-CP after co-incubation with thioTEPA [10]. Multiple CYP enzymes can catalyze CP 4-hydroxylation in vitro, including CYP2B6, CYP2C9 and CYP3A4 [12], with CYP2B6 playing the major role [13]. Indeed, thioTEPA has been identified as a specific inhibitor of human CYP2B6 activity characterised by *S*-mephenytoin-*N*-demethylation [14], but the mechanism of inhibition has not been investigated in detail. Expression and function of CYP2B6 is highly variable [15] and thus the contribution of CYP2B6 for CP-hydroxylation depends on individual expression levels and genotypes [16]. Several mutations have been identified which affect both expression and catalytic activity [15,16]. Although there are conflicting results with regard to dose-dependent clearance of thioTEPA [1], several studies using high dose chemotherapy protocols demonstrated an inverse relationship between clearance and dose [17,18], indicating either a saturable step in elimination or enzyme inhibition. In contrast to reversible enzyme inactivation, the loss of enzyme activity caused by irreversible inactivation persists even after elimination of the inhibitor, and de novo biosynthesis of new enzyme is the only means by which activity can be restored. Clinical and pharmacokinetic consequences of irreversible drug inhibition are thus quite complicated, depending on the duration, dose and frequency of administration [19]. In addition, many drugs which inhibit their own metabolism are mechanism-based inhibitors of CYPs [20]. In this study we investigated the mechanism of inhibition of the human CYP2B6 by thioTEPA using the specific probe drug, bupropion [21,22]. The results demonstrate that inhibition is mechanism-based, contrary to present knowledge [14]. The findings have implications for the clinical use and for application protocols in chemotherapy.

2. Materials and methods

2.1. Chemicals

ThioTEPA was obtained from Wyeth Pharma GmbH. $NADP^+$, NADPH, diethyldithiocarbamate (DDC), sulfaphenazole, superoxide dismutase (SOD), *N*-acetylcysteine (*N*-Ac), DMSO, gluthathione (GSH), 7-ethoxycoumarin, coumarin, umbelliferone, quinidine and sodium hydrosulfite were purchased from Sigma. Furafylline was a kind gift from U. Fuhr (University of Cologne, Germany). Glycerine was purchased from Roth and Emulgen 911 was provided by Kao-Atlas. Glucose-6-phosphate (GP) was obtained from Roche Diagnostics GmbH. Glucose-6-phosphate-dehydrogenase (GPD) was purchased from Calbiochem. Ketoconazole was obtained from Ultrafine Chemicals. *S*-Mephenytoin was a kind gift from U.A. Meyer (Biozentrum, Basel, Switzerland). Propafenone, verapamil and metabolites were obtained from Knoll. Bupropion hydrochloride, hydroxybupropion hydrochloride and [$^2H_3$]-hydroxybupropion hydrochloride, 4′-hydroxymephenytoin, [$^2H_3$]-4′-hydroxymephenytoin and [$^2H_5$]-nirvanol were synthesized as described by Richter et al. [23].

2.2. Cytochromes P450 and human liver microsomes

Recombinant CYPs co-expressed with NADPH-P450 oxidoreductase (OR) in insect cells (supersomes^{TM}) were purchased from BD Biosciences. Human liver microsomes were prepared from surgically removed liver tissue as described previously [15]. The study was approved by the Ethics Committees of the Medical Faculties of the Charité, Humboldt-University Berlin and of the University of Tübingen and written informed consent was obtained from each patient.

2.3. Determination of catalytic CYP activities

Details for each assay are described below. All assays were performed with recombinant CYPs (2.5–5 pmol) or human liver microsomes (50–100 μg protein) in a final volume of 250 μl with inhibitors as indicated. After equilibrating the reaction mixture at 37 °C for 3 min, preincubation with inhibitors was started by adding 25 μl of 10-fold concentrated NADPH-regenerating system (final concentrations, 5 mM $MgCl_2$, 4 mM glucose 6-phosphate, 0.5 mM $NADP^+$ and 4 U/ml glucose 6-phosphate dehydrogenase) and performed for the indicated times at 37 °C. Subsequently, enzyme reactions were started by the addition of substrate. Reactions were terminated and processed as described below.

Bupropion-hydroxylation [21,22] was performed with CYP2B6 or human liver microsomes at concentrations indicated in 0.1 M sodium phosphate buffer pH 7.4, and with clopidogrel (1 μM) as a CYP2B6 control inhibitor [23]. Enzyme reactions were carried out using 50 μM bupropion with different incubation times for different assays to allow a sensitive detection of inhibition. The reactions were stopped by adding 50 μl of 1N HCl. After addition of the internal standard d_3-OH-bupropion (100 pmol), the samples were centrifuged at 16,000 × *g* for 5 min. The supernatant was directly injected into the HPLC system. The metabolite hydroxy-bupropion was separated and detected by HPLC-ESI-mass spectrometry using a HPLC system (HP 1100, Agilent Technologies) equipped with a Prontosil-C18 AQ column (150 mm × 3 mm, 3 μM particle size, Bischoff) and a mass spectrometer (Agilent Technologies). Elution was

performed with a gradient of 16% of 1% acetic acid in water (v/v) and 84% of 1% acetic acid in acetonitrile (v/v) to 45%/55% from 0 to 16 min.

The dynamic range for detection of hydroxybupropion was 1–500 pmol per incubation and assay accuracy over the calibration range was <8%. Formation of hydroxybupropion was linear with time up to 30 min and linear with protein between 5 and 200 μg of microsomal protein.

All other assays for enzymatic activities of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 were performed as previously described by Richter et al. [23]. Table 1 shows an overview for the different assay conditions.

2.4. Inhibition studies in human liver microsomes

Human liver microsomes (50–100 μg protein), bupropion (500 μM) and thioTEPA (0.1–10 μM) were equilibrated in 0.1N sodium phosphate buffer, pH 7.4, at 37 °C for 3 min. After addition of 25 μl NADPH-regenerating system, reactions were allowed to proceed for 15 min and analyzed as described for the bupropion hydroxylase assay. The effect of nucleophilic trapping agents (10 mM gluthathione or *N*-acetylcysteine) or scavengers of reactive oxygen species (0.1% DMSO or 1000 Units of superoxide dismutase) was tested by adding these compounds at the indicated concentrations prior to incubation with inhibitors. Substrate protection was analyzed accordingly with 7-ethoxycoumarin (500 μM) and 5 μM thioTEPA (controls without inhibitors) in the incubation mixture and determination of residual bupropion hydroxylase activity as described above.

2.5. Dialysis experiments

CYP2B6 supersomes (0.1 μM cytochrome P450) were incubated with or without thioTEPA (50 μM) and with or without NADPH-regenerating system for 15 min as described for the inactivation assay. The samples were then immediately dialyzed against 0.1 M sodium phosphate buffer, pH 7.4 (3 × 2 l, 2 h each) at 4 °C in QuixSep micro dialyzer capsules and a regenerated cellulose tubular membrane with molecular mass cut-off of 12 kDa (Roth GmbH). Bupropion hydroxylase activity was then determined with 500 μM bupropion and 15 min incubation time as described.

2.6. P450 reduced CO-difference spectroscopy

Recombinant CYP2B6 and oxidoreductase (0.74 μM), were incubated in 0.1N sodium phosphate buffer pH 7.4 in the presence or absence of thioTEPA (100 μM). Inhibition was started by adding NADPH-regenerating system. Controls were incubated without NADPH-regenerating system. The reactions were allowed to proceed for 15 min and were then stopped with 1.75 ml quenching buffer (0.1 M sodium phosphate buffer, pH 7.4, 10% glycerol, 0.5% Emulgen 911). Dithionite was added, the samples were gently bubbled with CO for 15 s and the reduced carbonyl spectrum was recorded between 400 and 500 nm on an Agilent 8453 UV–VIS spectrophotometer (Agilent Technologies). Before termination with quenching buffer, 25 μl samples were taken to determine bupropion hydroxylase activity as described above.

2.7. Kinetic inhibition studies with ThioTEPA

All incubations were carried out at 37 °C with either recombinant CYP2B6 + OR supersomes (BD Biosciences) or 100 μg human liver microsomes. The samples were equilibrated with different concentrations of thioTEPA (ranging from 0.1 to 10 μM) for 3 min at 37 °C and after the addition of NADPH-regenerating system, the samples were incubated for 0–15 min as indicated. Subsequently, 25 μl of the preincubation mixture was transferred to 225 μl of enzyme activity assay mixture, consisting of 0.1 M sodium phosphate buffer, pH 7.4, 500 μM bupropion and NADPH-regenerating system, pre-warmed to 37 °C. After 6 min of incubation, the reactions were stopped with 50 μl 1N HCl. After the addition of 100 pmol of the internal standard, d_3-hydroxy-bupropion, the samples were vortexed and

Table 1
Summary of incubation conditions for cytochrome P450 marker assays

CYP	Reaction[a]	Substrate concentration (μM)	Control inhibitor	Inhibitor concentration (μM)	Residual activity[b,c] (%)
1A2	7-Ethoxycoumarin *O*-deethylation	10	Furafylline	10	8.4
2A6	Coumarin 7-hydroxylation	200	Diethyldithiocarbamate	100	30.7
2B6	Bupropion-hydroxylation	50	Clopidogrel	1	2.4
2C8	Verapamil O-demethylation	100	Ketoconazole	100	n.d.[b]
2C9	*S*-Mephenytoin *N*-demethylation	10	Sulfaphenazole	10	34.7
2C19	*S*-Mephenytoin 4′-hydroxylation	200	Ketoconazole	100	41.7
2D6	Propafenone 5-hydroxylation	2	Quinidine	5	40.7
2E1	7-Ethoxycoumarin *O*-deethylation	200	Diethyldithiocarbamate	1	1.2
3A4	Verapamil *N*-demethylation	100	Ketoconazole	100	18.2

[a] Assay conditions were as described for the determination of catalytic CYP activities under Section 2.
[b] Results are means of two to four independent experiments; n.d., not detectable.
[c] Control activity without inhibitor was set at 100%.

centrifuged for 5 min (16,000 × *g*). The supernatant was directly injected into the HPLC-system (HP 1100, Agilent Technologies) and analyzed as described for the bupropion-hydroxylation assay.

2.8. Data analysis

Enzyme kinetic data were analyzed according to the method of Silverman [24]. The half-time of enzyme inactivation ($t_{1/2}$) was calculated from the initial slopes of the remaining enzyme activity, plotted semi-logarithmically against the preincubation time. The half-time of enzyme inactivation thus obtained was plotted against the reciprocal of the respective thioTEPA concentration (Kitz–Wilson plot). The concentration required for half-maximal inactivation (K_I) and the maximum inactivation rate constant (K_{inact}) were determined from the intercepts on the abscissa and ordinate, respectively.

3. Results

3.1. Effect of thioTEPA on P450 monooxygenase activities

To determine the selectivity of inhibition by thioTEPA towards catalytic activities representing the most relevant drug metabolizing CYPs, recombinant enzymes coexpressed with OR were analysed with appropriate assays using CYP isoform-specific inhibitors as positive controls (Table 1). Bupropion-hydroxylation, as a specific marker reaction for CYP2B6 [21,22] was inhibited by almost 90% at 10 μM concentration. Incubations of human liver microsomes with 10 μM thioTEPA and NADPH-regenerating system also reduced bupropion hydroxylase activity by up to 87% (data not shown). Control incubations with NADPH-regenerating system in the absence of inhibitor showed an approximate 20% loss of enzyme activity during 30 min incubation time which was not prevented by the addition of catalase (data not shown). CYP2C9 was inhibited by 24%, CYP2A6 by 17% and CYP2E1 by 13% (Fig. 1). Other CYPs were not markedly affected at this concentration.

3.2. Mechanism-based inactivation of CYP2B6 by thioTEPA

Kinetic experiments in human liver microsomes revealed unusual inhibition kinetics not explained by reversible inhibition mechanisms (data not shown). In particular, we observed non-competitive inactivation of bupropion-hydroxylation and increased inhibition with longer preincubation times with thioTEPA. To investigate whether the inactivation was the result of an irreversible mechanism, we performed dialysis experiments in which CYP2B6 supersomes were incubated with thioTEPA in the presence or absence of

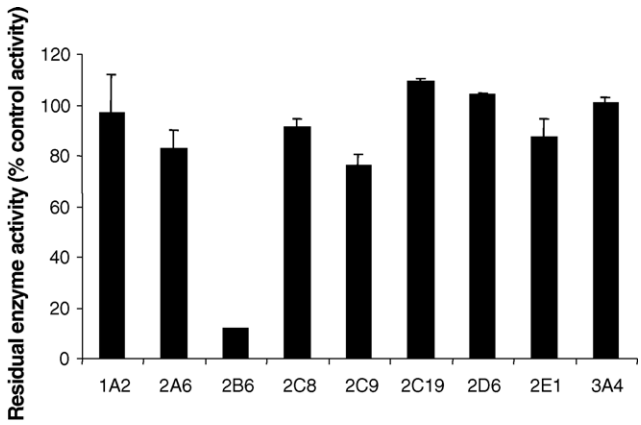

Fig. 1. Selectivity of inhibition by thioTEPA in recombinant CYP enzymes. Cytochrome P450 enzymes recombinantly coexpressed with OR in insect cells (supersomes™) were incubated with 10 μM of thioTEPA. CYP activities were determined using appropriate marker drug assays as detailed in Table 1. For each CYP, control activity measured in the absence of inhibitor was set at 100%. Data represent the means of two independent measurements.

NADPH-regenerating system. As shown in Fig. 2, extensive dialysis of samples incubated with inhibitor (50 μM) and NADPH-regenerating system did not lead to recovery of bupropion-hydroxylase activity whereas samples incubated with inhibitor in the absence of NADPH-regenerating system regained almost full activity compared to controls without inhibitor. This suggested that NADPH-dependent activation of thioTEPA to a reactive intermediate by CYP2B6 was required for enzyme inactivation. Fig. 3 shows that inhibition of CYP2B6 by thioTEPA was attenuated by the presence of the alternative active site ligand 7-ethoxycoumarin, indicating competition at the active site. Taken together these results demonstrated unequivocally the irreversible and mechanism-based inhibition type by thioTEPA.

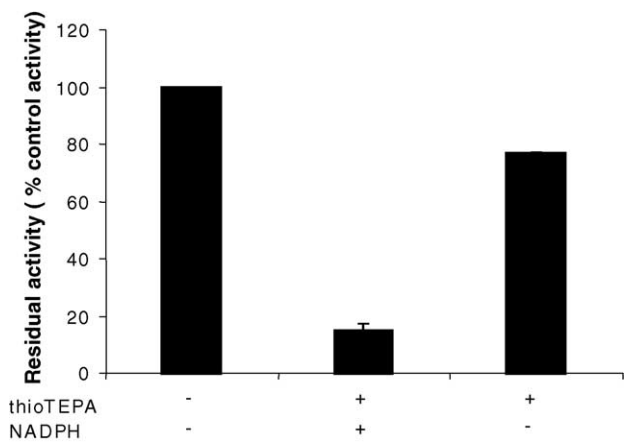

Fig. 2. Mechanism-based irreversible inhibition of bupropion-hydroxylation. CYP2B6 supersomes (Gentest), 0.1 μM were incubated for 15 min with NADPH-regenerating system and 50 μM of thioTEPA. Controls were incubated without inhibitor and without NADPH-regenerating system, as indicated. After extensive dialysis, residual bupropion-hydroxylase activity was determined and compared to the dialysed control samples which were set to 100%. Samples were carried out in duplicate. The means of two independent experiments is shown.

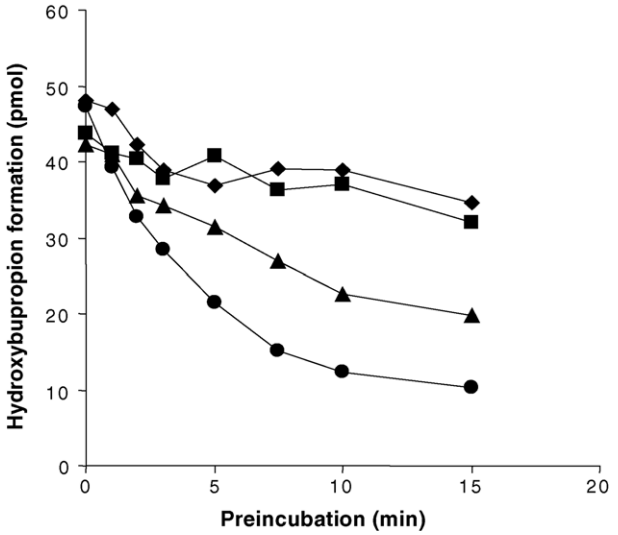

Fig. 3. Substrate protection by alternative active site ligands. Human liver microsomes (100 μg) were incubated with 5 μM thioTEPA in 0.1N sodium phosphate buffer, pH 7.4, at 37 °C for 3 min in the presence (▲) or absence (●) of 7-ethoxycoumarin (500 μM). Controls were incubated in the absence of thioTEPA with (■) and without 7-ethoxycoumarin (◆). After addition of 25 μl NADPH-regenerating system, reactions were allowed to proceed for indicated times and residual bupropion-hydroxylase activity was determined as described in Section 2. The difference between the inhibition curves obtained in the presence and absence of 7-ethoxycoumarin was statistically significant for preincubation times >3 min ($p < 0.001$, Anova-test). Results represent the means of two independent measurements.

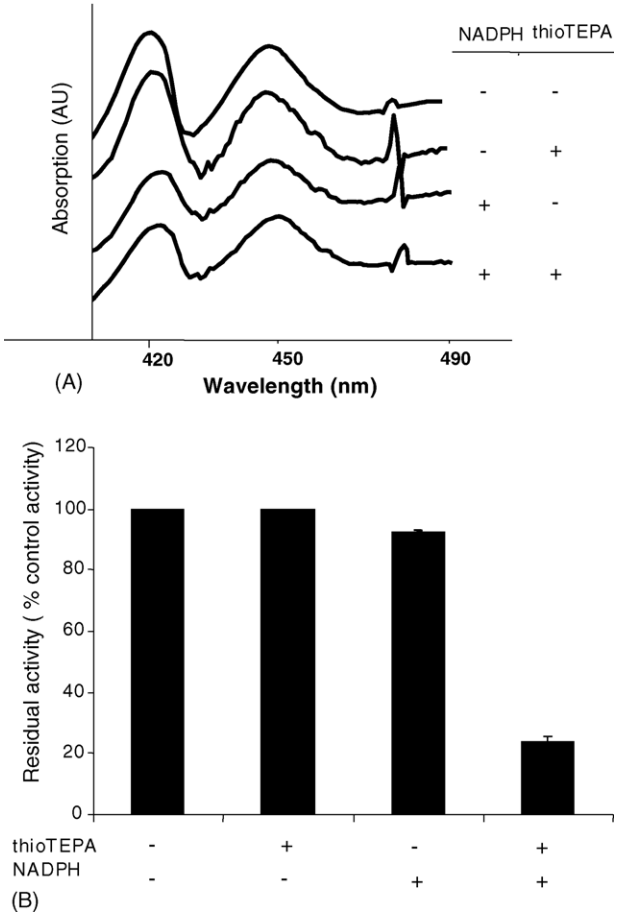

Fig. 4. (A) Reduced CO-difference spectra of CYP2B6. Recombinant CYP2B6 and OR (0.74 μM) were incubated for 15 min in the presence or absence of thioTEPA and NADPH-regenerating system, respectively. Finally, the reduced CO-difference spectra were recorded as described in Section 2 (AU; arbitrary units). (B) Bupropion hydroxlase activities corresponding to the reduced CO-difference spectra shown in Fig. 4A. Residual bupropion-hydroxylation was determined after incubation with or without thioTEPA and NADPH as indicated. Data represent the means of two independent measurements.

Inactivation occurs prior to release of the inactivator from the active site because addition of the nucleophilic trapping agents gluthathione (10 mM) and *N*-acetylcysteine (10 mM) to the incubation mixture did not prevent inactivation with thioTEPA. Furthermore, the reactive oxygen scavengers DMSO (0.1%) and superoxide dismutase (1000 Units) had no measurable effect on CYP2B6 inhibition caused by thioTEPA (data not shown) which demonstrates that no autoinactivation step by peroxides is involved in inhibition. To analyze whether inhibition of bupropion-hydroxylase activity was due to destruction of the heme mojety, we recorded reduced CO-difference spectra of recombinant CYP2B6 incubated with thioTEPA in the presence or absence of NADPH-regenerating system. There was no visible destruction of the heme compared to controls without inhibitor as shown in Fig. 4A. Fig. 4B shows the corresponding bupropion-hydroxylase activities. Incubation with NADPH-regenerating system or with thioTEPA and NADPH-regenerating system caused an approximate 20% loss of spectrally detectable P450 (Fig. 4A). This is consistent with the finding that CYP2B6 activity decreases with time when incubated with NADPH-regenerating system alone.

3.3. Kinetic analysis of inactivation of bupropion-hydroxylation

Detailed kinetic investigation of mechanism-based inhibition reactions requires separation of the inactivation step from observation of substrate metabolism, which is usually achieved by dialysis or by diluting the incubation mixtures. We, therefore, determined residual bupropion-hydroxylase activity in 10-fold diluted incubation mixtures after preincubation with inhibitor (see Section 2). Inactivation increased with longer preincubation time and higher thioTEPA concentrations in both cases, proceeded very rapidly and showed saturation. The microsomal inactivation kinetics of thioTEPA at various concentrations of inhibitor is presented in Fig. 5A. Inactivator concentration for half-maximal inactivation (K_I) due to mechanism-based enzyme inhibition was calculated to be 3.8 μM by transferring the data into a Kitz–Wilson plot (insert in Fig. 5A). The inactivation process was of a non-pseudo-first-order type. K_{inact}, the maximal rate of inactivation at saturating concentrations of thioTEPA was 0.16 min^{-1}. Accordingly, the time required for half of the enzyme molecules to be inactivated ($t_{1/2}$) was 4.3 min. Fig. 5B shows the inactivation of recombinant CYP2B6 by thioTEPA, which was

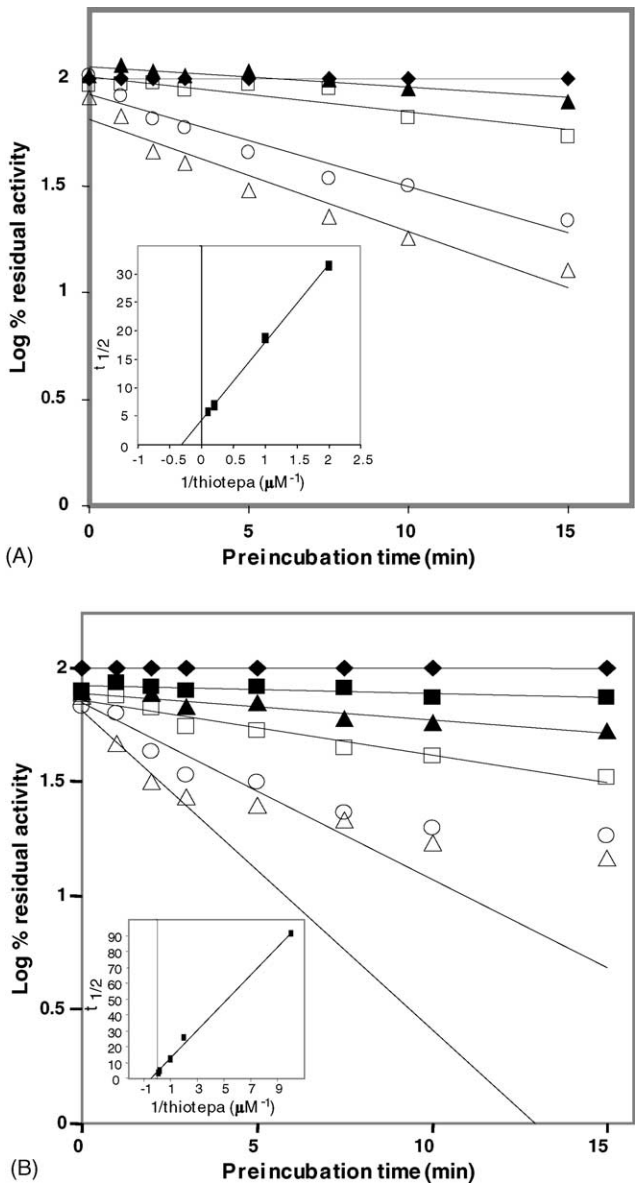

Fig. 5. Kinetics of inactivation of microsomal (A) and recombinant (B) bupropion-hydroxylation by different concentrations of thioTEPA. For details on incubation and determination of activity see Section 2. Human liver microsomes (100 μg) or supersomes™ (5 pmol) from insect cells expressing CYP2B6 were incubated for the indicated times in the presence of NADPH-regenerating system and thioTEPA as indicated: (◆) 0 μM; (■) 0.1 μM; (▲) 0.5 μM; (□) 1 μM; (○) 5 μM; (Δ) 10 μM and subsequent residual enzyme activity was determined. The inserts show the corresponding Kitz–Wilson plots.

very similar to that in human liver microsomes, revealing a K_I of 2.2 μM and a K_{inact} of 0.17 min^{-1} at $t_{1/2}$ of 4.1 min.

4. Discussion

The cytostatic agent, thioTEPA, has previously been shown to be a specific inhibitor of CYP2B6 [14]. In their work, the authors concluded that the inhibition type was non-competitive, but they did not assess the actual mechanism of inhibition. The present in vitro study provides additional data on the cytochrome P450 interaction specificity of thioTEPA and demonstrates that it acts as a potent mechanism-based inhibitor of human CYP2B6. These conclusions are based on experiments with human liver microsomes and with recombinant CYPs using bupropion-hydroxylation as CYP2B6-selective marker enzyme activity [21,22]. This is in contrast to the study by Rae et al. [14] who performed inhibition experiments using *S*-mephenytoin as a CYP2B6 substrate, which has limited selectivity for CYP2B6 [25]. Our specific findings were that inhibition of bupropion-hydroxylation by thioTEPA was (1) time- and concentration-dependent; (2) NADPH-dependent; (3) irreversible; (4) not affected by scavengers of reactive oxygen species or by nucleophilic trapping agents; (5) reduced by the presence of an alternative active site ligand; and (6) not associated with a decrease in spectrally detected P450. Investigation of the inhibition profiles of thioTEPA included the nine P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, representing the major drug-metabolizing CYPs in human liver. Only CYP2A6, CYP2C9 and CYP2E1 were inhibited by thioTEPA in addition to CYP2B6 but their inhibition did not exceed 25%. Rae et al. [14] found that inhibition of other CYPs did not exceed 20 and 15% in human liver microsomes and recombinant CYPs, respectively. We confirmed the weak inhibition of CYP2E1, but in addition we found a 24% loss of CYP2C9 activity by thioTEPA (10 μM) and a 17% decreased activity of CYP2A6, which was not addressed in the previous study [14]. Time- and concentration-dependent inactivation of bupropion-hydroxylase activity in human liver microsomes and in recombinant CYP2B6-expressing insect cell membranes proceeded with almost identical characteristics. Inactivation proceeded very rapidly within the first few minutes and slowed down thereafter, probably due to consumption of the inhibitor by metabolism. The irreversible and NADPH-dependent nature of inhibition was demonstrated by dialysis experiments. After dialysis, incubations containing thioTEPA but no NADPH showed about 80% of the activity of those lacking both thioTEPA and NADPH. Potent inhibition of bupropion-hydroxylation occurred only in the presence of NADPH during incubation with the inhibitor, strongly suggesting that catalytic turnover was required for inhibition (Fig. 2). Further evidence for the involvement of the CYP2B6 active site in the activation of thioTEPA is provided by the observation that an alternative active site ligand effectively attenuated the inhibitory potency. These data are in agreement with the finding that thioTEPA is a mechanism-based inhibitor of rat CYP2B1, the orthologous form of human CYP2B6, although with lower potency reflected by a Ki of 24 μM [2]. Spectral analysis of recombinant CYP2B6 did not indicate any destruction of the heme moiety caused by thioTEPA during inhibition (Fig. 4A), suggesting that alkylation of the apoprotein is the major event leading

to inhibition. This has accordingly been shown for the structurally similar insecticide parathion, which causes CYP inhibition by covalent binding of elemental sulfur to cysteine residues of P450 of the rat [26].

In vivo metabolism of thioTEPA in rat was found to follow biphasic kinetics after a single dose of the drug [3]. In humans the in vivo clearance of thioTEPA decreases at higher dosages suggesting a saturable step in elimination [17,18]. As CYP2B6 is one of the thioTEPA metabolizing enzymes in human liver [4], it is plausible to assume suicide inhibition of CYP2B6 as the cause for saturable kinetics.

At least one clinically relevant drug interaction involving thioTEPA has been described until now. The anticancer drug was shown to cause a significant reduction in the plasma levels of the active metabolite of the CYP2B6 substrate CP when administered first [11]. Because of the irreversible nature of CYP2B6 inhibition, restoration of complete enzyme activity must be expected to depend on de novo enzyme synthesis. Support for this assumption may be provided by a study where simultaneous administration of CP and thioTEPA on a multiple-injection schedule resulted in sub-additive response and the best result was obtained with an interval between the administration of the two drugs of 8 h [27]. To our knowledge, the half-life of CYP2B6 in human liver is not known but pharmacokinetic modeling predicted a value of 24 h [28]. CP is a known inducer of CYP2B6 [29] and therefore several studies investigated the extent of CYP2B6 induction by measuring 4-hydroxy-CP plasma-levels. Two similar studies demonstrated that thioTEPA reverses the induction of 4-hydroxy-CP plasma-levels. Patients who received only CP had a 54.7% increase in AUC levels of 4-hydroxy-CP from day one to day two [30] whereas patients who received a high dose chemotherapy in combination with thioTEPA and carboplatin had no changes in 4-hydroxy-CP plasma concentrations [28]. Another clinically relevant implication of irreversible CYP inhibition is the initiation of immunoallergic reactions against epitopes generated through CYP alkylation as described for CYP2C9 and its irreversible inhibitor, ticrynafen [31]. Some patients treated with thioTEPA develop skin toxicity with pruritus and dermatitis [32]. Because CYP2B6 was shown to be expressed in human skin, adverse side effects of thioTEPA affecting skin may be related to alkylation of CYP2B6 [33,34].

In conclusion, we found that thioTEPA is a highly potent, irreversible inhibitor of CYP2B6. We provided strong evidence that inhibition involves a mechanism-based process presumably resulting in the alkylation of the apoprotein. Because the pharmacokinetic consequences of irreversible inhibition are substantially different compared to those of competitive inhibition of drug metabolism, these findings should be carefully considered in optimizing chemotherapy and minimizing drug interactions. In addition, these findings have implications for the use of thioTEPA in combination with other CYP2B6 substrates than CP.

Note added in proof

Since submission of this manuscript, Harleton et al. [35] also reported the mechanism-based inhibition of CYP2B6 by thio TEPA, although with considerably lower potency. It should be noted that their study was exclusively based on genetically modified CYP2B6 expressed in *E. coli*.

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