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Inhibition of CYP2E1 catalytic activity in vitro by *S*-adenosyl-L-methionine

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Abstract

The objective of this work was to evaluate the possible in vitro interactions of *S*-adenosyl-L-methionine (SAM) and its metabolites *S*-(5′-Adenosyl)-L-homocysteine (SAH), 5′-Deoxy-5′-(methylthio)adenosine (MTA) and methionine with cytochrome P450 enzymes, in particular CYP2E1. SAM (but not SAH, MTA or methionine) produced a type II binding spectrum with liver microsomal cytochrome P450 from rats treated with acetone or isoniazid to induce CYP2E1. Binding was less effective for control microsomes. SAM did not alter the carbon monoxide binding spectrum of P450, nor denature P450 to P420, nor inhibit the activity of NADPH-P450 reductase. However, SAM inhibited the catalytic activity of CYP2E1 with typical substrates such as *p*-nitrophenol, ethanol, and dimethylnitrosamine, with an IC_{50} around 1.5–5 mM. SAM was a non-competitive inhibitor of CYP2E1 catalytic activity and its inhibitory actions could not be mimicked by methionine, SAH or MTA. However, SAM did not inhibit the oxidation of ethanol to α-hydroxyethyl radical, an assay for hydroxyl radical generation. In microsomes engineered to express individual human P450s, SAM produced a type II binding spectrum with CYP2E1-, but not with CYP3A4-expressing microsomes, and SAM was a weaker inhibitor against the metabolism of a specific CYP3A4 substrate than a specific CYP2E1 substrate. SAM also inhibited CYP2E1 catalytic activity in intact HepG2 cells engineered to express CYP2E1. These results suggest that SAM interacts with cytochrome P450s, especially CYP2E1, and inhibits the catalytic activity of CYP2E1 in a reversible and non competitive manner. However, SAM is a weak inhibitor of CYP2E1. Since the K_i for SAM inhibition of CYP2E1 activity is relatively high, inhibition of CYP2E1 activity is not likely to play a major role in the ability of SAM to protect against the hepatotoxicity produced by toxins requiring metabolic activation by CYP2E1 such as acetaminophen, ethanol, carbon tetrachloride, thioacetamide and carcinogens.

Keywords: *S*-adenosyl-L-methionine; Cytochrome P450; CYP2E1; Binding spectrum; Substrate oxidation; HepG2 cells

1. Introduction

S-adenosyl-L-methionine (SAM) is the principal biological methyl donor, the precursor of aminopropyl groups utilized in polyamine synthesis and, in the liver, SAM is also a precursor of GSH through its convertion to cysteine via the *trans*-sulfuration pathway. The liver plays a central role in the homeostasis of SAM as the major site of its synthesis and degradation [1]. Impairment of SAM synthesis is believed to play an important role in hepatic injury induced by various agents. Exogenous administration of SAM can protect against injury induced by agents including ethanol [2], CCl_4 [3], acetaminophen [4], thioacetamide [5], bromobenzene [6] and cyclosporin A [7]. The beneficial actions of SAM have been ascribed mainly to its methylation capacity linked to membrane fluidization, and/or increased GSH synthesis [1].

Cytochrome P450 proteins catalyze the majority of drug biotransformations and play a pivotal role in several homeostatic mechanisms. Many hepatotoxins, including those mentioned above, require metabolic activation by liver cytochrome P450 enzymes, particularly CYP2E1, to form reactive, toxic metabolites that in turn produce liver injury in experimental animals and humans [8–13].

Any treatment that suppresses P450-mediated production of reactive metabolites could protect against

Abbreviations: SAM, *S*-adenosyl-L-methionine; POBN, *N-tert*-Butyl-α-(4-pyridyl)nitrone *N*′-oxide; SAH, *S*-(5′-Adenosyl)-L-homocysteine; MTA, 5′-Deoxy-5′-(methylthio)adenosine; HER, α-hydroxyethyl radical; 7-ECOD, 7-ethoxycoumarin *O*-deethylase; PNP, *p*-nitrophenol; NDMA, dimethylnitrosamine; 7-MFC, 7-methoxy-4-trifluoromethylcoumarin; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; DBF, dibenzylfluorescein; DDTC, diethydithiocarbamate; TCA, trichloroacetic acid; E47 cells, HepG2 cell line derived after transfection with pCI-neo vector containing the human CYP2E1 cDNA.

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chemical-induced liver injury [14]. Interaction of cytochrome P450 enzymes with SAM, and suppression of P450-catalyzed activity as a possible mechanism that could be involved in the protection against hepatotoxicity by SAM has not been evaluated experimentally, although the protection from CCl_4-induced hepatotoxicity in cultured rat hepatocytes was suggested to depend on a possible interaction of SAM with the intracellular drug-metabolizing enzyme systems [15]. However, it is important to note that SAM also protects against liver toxicity by agents that do not require P450 activation, such as D-galactosamine [6,16].

The objective of this work was to evaluate the possible interaction of SAM and its metabolites *S*-(5′-Adenosyl)-L-homocysteine (SAH), 5′-Deoxy-5′-(methylthio)adenosine (MTA) and methionine with cytochrome P450 enzymes, in particular CYP2E1. Studies were carried out to evaluate binding to cytochrome P450, and effect on the P450-dependent metabolism of substrates, and on the P450-dependent generation of reactive radical species such as the α-hydroxyethyl radical (HER).

2. Materials and methods

2.1. Chemicals

All chemicals were of the highest quality commercially available. Ethanol 95% was from Pharmaco Products, Inc. Protein concentration was measured using the BioRad DC Protein assay. HCl and acetonitrile were from Fisher Scientific. The CYP2E1 and CYP3A4 assay kits were from BD Biosciences. Other chemicals used were from Sigma-Aldrich.

2.2. Rat liver microsomes

Male, Sprague Dawley rats, weighing 150 g, were treated for 14 days with 1% (v/v) acetone in the drinking water to induce CYP2E1 [17]. Controls received water. Rats were starved overnight prior to being killed. Minced livers were homogenized in 0.15 M potassium phosphate buffer pH 7.4 (5 mL/g), and centrifuged at 12,000 × *g* for 15 min. The supernatant was centrifuged at 105,000 × *g* for 90 min. Microsomal pellets were washed twice in the resuspension buffer and centrifuged at 105,000 × *g* for 60 min. Microsomal pellets were resuspended in 50 mM potassium phosphate buffer pH 7.4, at 20 mg protein/mL, stored at −80 °C, and used within 3 months [18]. Liver microsomes from rats treated with isoniazid and the appropriate control microsomes were obtained from In Vitro Technologies.

2.3. Purification of SAM

Commercially available SAM (around 70% pure) is contaminated with its degradation products (e.g. SAH and MTA), and solvents (ethanol, methanol, and water). Considering this, SAM was purified by C18 reverse phase chromatography prior to use, according to Fujioka and Ishiguro, (1986) [19]. 1 mL of an aqueous solution of SAM was applied to a C18 cartridge (Sep Pak, Waters Corporation), previously conditioned with acetonitrile and flushed with water. The sample was eluted with water, and the fractions with absorbance at 254 nm were isolated. Samples were concentrated by partial lyophilization using a Speed Vac concentrator. Reverse phase HPLC of the eluate (described in Results) showed a unique peak at the retention time of SAM (27 min) and the absence of SAH and MTA (retention times of 23 and 51 min, respectively). Solvent (ethanol + methanol) concentration in the stock solution of SAM (100 mM) was reduced to 0.1 mM as assayed by an enzymatic method using alcohol dehydrogenase. SAM metabolites (SAH, MTA) were from Sigma-Aldrich, and used without further purification (>99% pure).

2.4. Concentration of SAM

SAM was quantified by HPLC as described by She et al. (1994) [20], using a Shimadzu SPD-10A UV–vis detector operating at 254 nm. Samples were mixed 1:2 with 0.4 M $HClO_4$, filtered, and applied directly for HPLC analysis. A TSKgel ODS column (15 cm × 4.6 mm i.d., Tosoh Corporation) was used, with a mobile phase that consisted of 40 mM $NH_4H_2PO_4$, 8 mM 1-heptanesulfonic acid and 18% (v/v) methanol, pH adjusted to 3.0 with HCl. The concentrations of SAM used in the experiments were linear with the areas under the HPLC chromatogram.

2.5. Binding spectra

Binding of SAM or DMSO to cytochrome P450 in rat liver microsomes was studied by difference UV–vis spectroscopy using 0.5 μM P450 in 0.1 M phosphate buffer (pH 7.4). The solution was equally divided between 2 cuvettes (1 mL/cuvette). After recording the baseline, aliquots of test substance were added to the sample cuvette, and the same volume of solvent was added to the reference cuvette. Difference spectra were recorded over a 350–450 nm range at room temperature, using a Shimadzu UV160U UV–vis spectrophotometer [21]. Binding in insect microsomes was studied following the same protocol, although using 0.1 μM P450 (due to the relatively low protein concentration in insect microsomes); high sensitivity was achieved by using a Perkin Elmer 557 double wavelength double beam spectrophotometer with slow scan speed, and using split cells to lower the contribution from the spectrum of SAM [21].

2.6. Cytochrome P450 content

The P450 concentration was measured as described by Omura and Sato (1964) [22].

2.7. Cytochrome b5 content

The content of cytochrome b5 was measured according to Ozols (1974) [23], measuring the change in absorbance at 423 nm ($A^{423}_{red} - A^{423}_{ox}$, $\varepsilon = 118\ mM^{-1}\ cm^{-1}$) which occurs upon reduction caused by the addition of solid dithionite.

2.8. NADPH cytochrome c reductase activity

The assay was performed at 25 °C in a 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 40 μM cytochrome c, and 60 μg/mL of microsomal protein. The reaction was initiated by the addition of 96 μM NADPH and the rate of cytochrome c reduction ($\Delta A_{550-541}$, $\varepsilon = 21\ mM^{-1}\ cm^{-1}$) was followed for 2 min [24].

2.9. SAM metabolism

Metabolism of SAM was studied at 37 °C in 100 mM phosphate buffer (pH 7.4) containing 1 μM P450 from acetone-treated rat liver microsomes, up to 2 mM SAM, and 1 mM NADPH. The incubation was stopped at the indicated times by addition of 2 volumes of 0.4 M $HClO_4$. The mixture was centrifuged at 10,000 × *g* for 20 min, and the resulting supernatant was filtered through a Millipore membrane (0.45 μm). Aliquots of the extracts were directly applied for HPLC analysis.

2.10. 7-Ethoxycoumarin-O-deethylase (7-ECOD) activity

The activity of 7-ECOD was measured according to Aitio (1978) [25]. The basic reaction was carried out in 100 mM phosphate buffer, pH 7.4, 50 μM 7-ethoxycoumarin and 0.5 mg microsomal protein/mL, and initiated by the addition of 1 mM NADPH. After 10 min of incubation at 37 °C, the reaction was stopped with TCA (final concentration of 3%). After centrifugation, 0.6 mL of the supernatant was mixed with 2.4 mL of 1.6 M glycine-NaOH, pH 10.3, and the fluorescence at 390/440 was measured.

2.11. PNP hydroxylation activity

The basic incubation system to assay PNP hydroxylation activity consisted of 100 mM potassium phosphate buffer, pH 7.4, plus 0.5 mg microsomal protein/mL, 0.1 mM PNP, and 1 mM NADPH. After 15 min incubation at 37 °C, the reaction was stopped with TCA (5% final concentration), samples were centrifuged, and 1 N NaOH final concentration was added to the supernatant. The $A_{510\ nm}$ was measured for each sample, and the concentration of product was determined from a calibration curve of *p*-nitrocatechol [26].

2.12. Ethanol oxidation

The production of acetaldehyde from ethanol by microsomes was assayed as previously described [27]. Reactions were carried out in center-well flasks containing 0.15 mL of 15 mM semicarbazide HCl in 180 mM potassium phosphate, pH 7.4, in the center well. The basic reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 50 mM ethanol, 1 mM sodium azide, 0.1 mM deferioxamine, and 50 μg of microsomal protein in a final volume of 0.1 mL. Reactions were started with 1 mM NADPH, and terminated by the addition of TCA to a final concentration of 4.5% (w/v). The sealed flasks were incubated overnight at room temperature to allow diffusion of acetaldehyde into the center well. The absorbance of the aldehyde–semicarbazone complex was determined at 224 nm.

2.13. NDMA oxidation

The oxidation of dimethylnitrosamine (NDMA) to formaldehyde was determined in a reaction system containing 0.1 M potassium phosphate buffer, 2 mM NDMA, 1 mM NADPH and microsomes from acetone-treated rats. The reaction was terminated with 5% TCA (w/v), and formaldehyde was determined by the Nash reaction. All values were corrected for zero time controls in which TCA was added prior to NADPH [28].

2.14. Human cytochrome P450 metabolism

Fluorometric assays developed by BD Biosciences, using microsomes derived from baculovirus infected insect cells engineered to express human CYP2E1 or CYP3A4 cDNA, were used. The reaction system consisted of a NADPH generating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 U/mL glucose-6-phosphate dehydrogenase), test inhibitory compounds, microsomes, and specific substrate (7-MFC or DBF for CYP2E1 or CYP3A4, respectively). After incubation at 37 °C, reactions were stopped and the fluorescence at optimal wavelength pairs was measured in a fluorescence plate reader.

2.15. α-Hydroxyethyl radical formation

The production of HER by rat liver microsomes in the presence of NADPH was assayed according to Rashba-Step et al. (1993) [29]. The basic microsomal system contained 100 mM phosphate buffer, pH 7.4, microsomes (usually 0.3 mg protein/mL), 30 mM POBN, 25 mM ethanol, and 1 mM NADPH. Experiments were carried out in the absence or presence of 1 mM sodium azide. EPR measurements to assay for the POBN-HER adduct were started 20–30 s after initiation of the reaction, and were carried out at room temperature, typically over a 5 min period. EPR measurements were performed on a Bruker ECS 106 spectrometer. Instrument settings were as follows: sweep width 100 G, sweep time 42 s, time constant 164 ms, modulation amplitude 0.7 G, microwave power 20 mW, modulation frequency 50 kHz.

2.16. CYP2E1 activity in intact cells

CYP2E1 enzymatic activity was determined in a HepG2 cell line derived after transfection with pCI-neo vector containing the human CYP2E1 cDNA (E47 cells) [30]. Incubations (in duplicate) were performed using 1 millon E47 cells/mL of Eagle's minimal essential medium without phenol red and fetal bovine serum, at 37 °C in a CO_2 incubator. Reactions were initiated by the addition of substrate at a concentration around the apparent K_m (5 μM 7-MFC in acetonitrile), and after the indicated intervals (0–2 h), cells were scraped and fluorescence of the suspension was determined at 409/530 nm [31].

2.17. Statistics

Data are expressed as mean ± standard error of the mean from 3 to 5 independent experiments. One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe was performed. A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Binding spectra

The interaction of SAM with liver microsomes from rats pretreated with acetone was followed by difference UV–vis spectroscopy. SAM produced a classical type II difference spectrum with a peak at around 425 nm and a trough around 390 nm (Fig. 1A). Apparent spectral dissociation constants (K_s) and ΔA_{max} were determined from Hanes plots representing [SAM] versus [SAM]/$\Delta A_{390–425}$; the K_s and ΔA_{max} for SAM was 0.6 mM and 0.020, respectively (Fig. 1B and Table 1). Microsomes from control rats either showed no detectable spectral interaction with SAM, or a weak interaction as reflected by a 10-fold higher dissociation constant than acetone-induced microsomes (Table 1). Microsomes from rats treated with isoniazid to induce CYP2E1 showed comparable spectral parameters as acetone-induced microsomes (Table 1). Solvents such as DMSO have been shown to interact with CYP2E1 to produce an inverse type I binding spectrum [32], and this was validated as a control (Table 1), to be compared to the SAM binding spectra. In order to determine whether the interaction between SAM and microsomes was reversible or not, washing experiments were carried out according to Schenkman et al. (1967) [33]. 2 mM SAM was addded to liver microsomes from rats treated with acetone, and the difference spectra was recorded. Then, the microsomal suspension was centrifuged, and the microsomal pellet was resuspended in buffer to the original volume. Control samples of microsomes used as reference were subjected to the same washing procedure. No spectral changes were observed after the washing procedure, suggesting that SAM binding is reversible by washing (data not shown).

Several analogs of SAM were studied for their possible binding to microsomes. Methionine, MTA, and SAH did not produce a difference spectra when incubated with liver microsomes from rats treated with acetone (data not shown).

Microsomes derived from baculovirus infected insect cells engineered to express human CYP2E1, available from BD Biosciences, also exhibited a type II binding spectrum with 2 mM SAM (0.1 μM P450) (Fig. 1C). Insect microsomes engineered to express human CYP3A4, or control insect microsomes which do not express any cytochrome P450, did not show a detectable binding spectrum at similar P450 (for CYP3A4) or protein (for controls) concentrations (data not shown).

3.2. P450-dependent metabolism

By HPLC, commercially available SAM eluted at a retention time of 27 min, and showed a purity of 82% (Fig. 2(i)), with SAH (retention time of 23 min) and MTA (retention time of 51 min), as the main impurities. Purified SAM (prepared as described in Section 2) showed an HPLC peak at the expected retention time of SAM (97% purity by HPLC, Fig. 2(ii); absence of SAH, MTA). After 60 min of incubation at 37 °C of SAM in buffer, around 10% of the SAM converted into MTA (detected at a retention time of 51 min) (Fig. 2(iii)). SAM is known to be non-enzymatically converted to MTA as a function of time [34]. To evaluate if SAM can be significantly metabolized by microsomes, SAM was incubated with acetone-treated rat liver microsomes in the presence of 1 mM NADPH (Fig. 2(iv), zero time control). After 60 min of incubation at 37 °C, around 10% of the original SAM was converted into MTA (Fig. 2(v)), a change similar to the non-enzymatic conversion. No SAH or other metabolites were detected as 90% of the original SAM was recovered, the same recovery as in the absence of microsomes. Thus, SAM was not metabolized actively by microsomes under these conditions.

3.3. Characterization of SAM interactions with liver microsomes

3.3.1. Effect of SAM on P450 activity

7-Ethoxycoumarin is a general P450 substrate, being metabolized by *O*-deethylation by several enzymes of the CYP1, CYP2 and CYP3 families [35]. 7-ECOD activity was assessed in order to evaluate the effect of SAM on P450 activity in acetone-treated rat liver microsomes. Preliminary experiments were conducted to ensure that the assay was linear with respect to microsomal protein concentration and incubation time (not shown). SAM inhibited 7-ECOD activity with an IC_{50} greater than

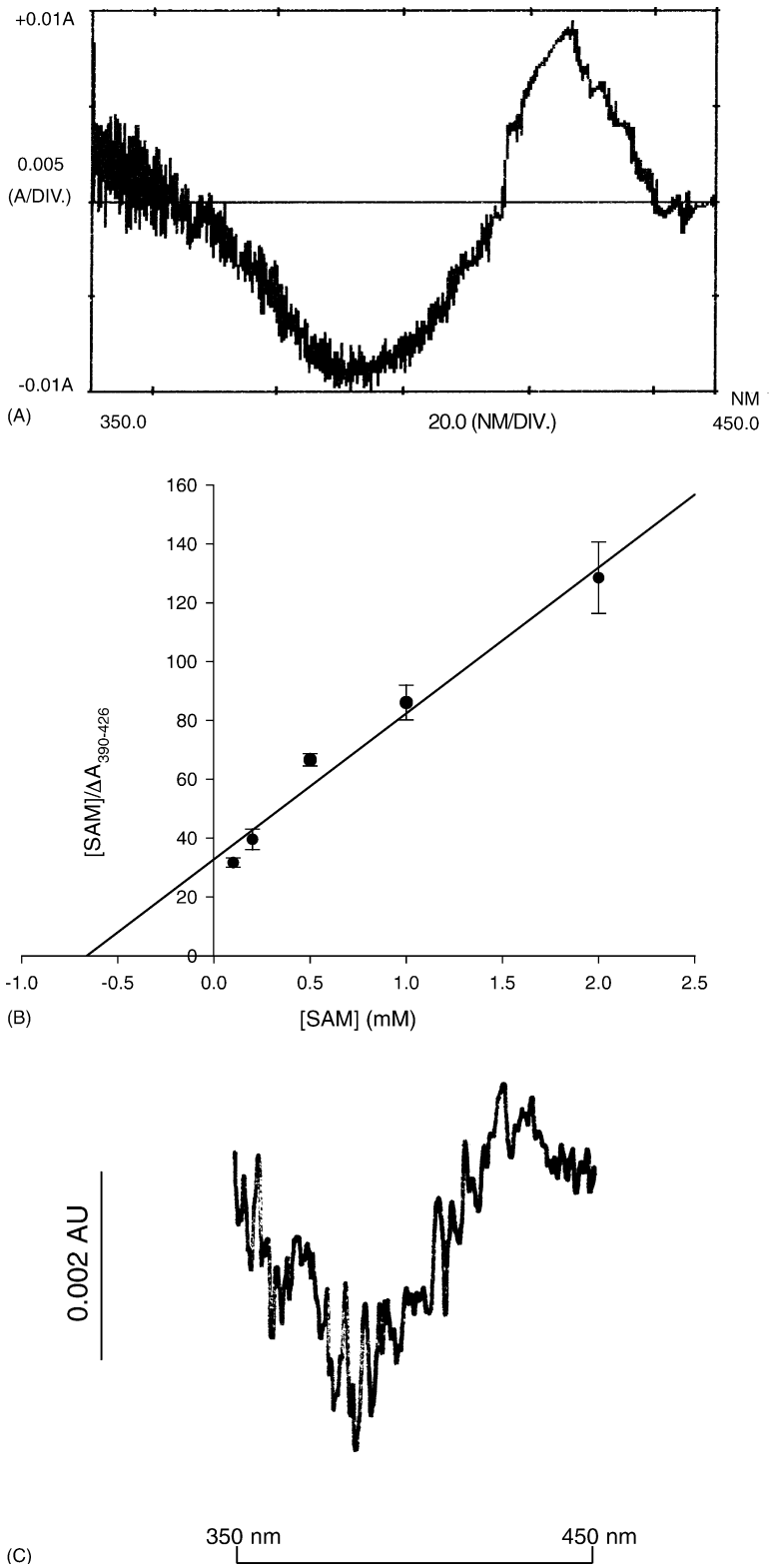

Fig. 1. Spectral changes caused by addition of SAM to microsomal membranes. (A) Difference spectra of 0.5 μM P450 from liver microsomes from rats treated with acetone, upon addition of 2 mM SAM to the sample cuvette. (B) Hanes plot ([SAM] versus [SAM]/$\Delta A_{390–426}$) using 0.5 μM P450 from acetone-treated rat liver microsomes. (C) Difference spectra (350–450 nm) of 0.1 μM P450 from insect microsomes engineered to express CYP2E1, upon addition of 2 mM SAM.

5 mM (36% inhibition at 5 mM SAM) (Fig. 3). Ketoconazole, a general P450 inhibitor, was much more effective as an inhibitor of 7-ECOD activity than was SAM (Fig. 3). At 5 mM, SAM did not interfere with a calibration curve using 7-hydroxycoumarin under the same conditions as in the inhibition assay. 5 mM SAM added after the incubation period did not produce any decrease in activity (not shown).

Table 1
Spectral interaction of SAM and DMSO with liver microsomes

Microsomes	Compound	Spectrum type (λ_{min}; λ_{max})	K_s (mM)	ΔA_{max}
Control 1	SAM	II (390; 425)	4.5	0.020
Control 2	SAM	n.d.	n.d.	n.d.
Acetone	SAM	II (390; 425)	0.6	0.020
Isoniazid	SAM	II (390; 425)	0.4	0.015
Control 1	DMSO	n.d.	n.d.	n.d.
Acetone	DMSO	Reverse I (385; 420)	7	0.017

n.d.: Non detectable. Difference spectra were obtained by addition of increasing amounts of SAM or DMSO to microsomal suspensions containing 0.5 μM P450 from rats either untreated (control 1, our laboratory prepared sample; control 2, commercially obtained sample), or treated with acetone or isoniazid. Spectral binding parameters were obtained from Hanes plots representing concentration vs. concentration/ΔA ($A_{max} - A_{min}$).

3.3.2. Effect of SAM on cytochrome P450 and cytochrome b5 content

A loss in the P450 CO spectrum is generally taken as an indicator of heme modification or heme denaturation or destruction [36]. SAM (up to 5 mM) did not produce a significant loss in the P450 carbon monoxide spectrum or of the content of cytochrome b5 (for cytochrome P450, 674 ± 36 pmol/mg prot with 0 mM SAM, versus 638 ± 62 pmol/mg prot with 5 mM SAM; for cytochrome b5, 397 ± 9 pmol/mg prot with 0 mM SAM, versus 398 ± 14 pmol/mg prot with 5 mM SAM). SAM did not increase the concentration of microsomal cytochrome P420 (data not shown). As positive controls, 3 M guanidine produced a complete loss of cytochrome P450 and conversion to cytochrome P420, and thermal denaturation (5 min 95 °C) produced a 50% reduction in cytochrome b5 content (not shown).

3.3.3. Effect of SAM on NADPH cytochrome c reductase

NADPH cytochrome P450 reductase transfers electrons from NADPH to cytochrome P450s, and in some cases the inhibition of P450 activity by certain chemicals is mediated via blocking of electron transfer from NADPH-P450 reductase [37]. SAM (up to 5 mM) did not show any significant changes of NADPH-cytochrome c reductase activity (96 ± 15 nmol/min/mg prot with 0 mM SAM, versus 95 ± 20 nmol/min/mg prot with 5 mM SAM); a positive control (100 μM diphenylene iodonium) significantly inhibited the activity (−63%, data not shown).

3.4. Inhibition of CYP2E1 in rat liver microsomes

The effect of SAM on CYP2E1 catalytic activity was studied in liver microsomes from rats treated with acetone, using several substrates known to be effectively metabolized by CYP2E1 such as PNP, ethanol and NDMA.

For the determination of the IC_{50} by SAM in the PNP hydroxylation assay, microsomes (0.5 mg protein/mL) were incubated in the presence of 0–2 mM SAM, 1 mM NADPH, and 100 μM PNP. Reactions were conducted under initial rate conditions (product formation was linear with microsomal protein and incubation time, and the consumption of the substrate was less than 20%) (not shown). SAM inhibited PNP hydroxylation with an IC_{50} of around 1.5 mM (Fig. 4A). SAM (2 mM) did not significantly affect the calibration curve performed with 4-nitrocatechol under the same conditions as in the inhibition assay (data not shown). SAM was, however, less effective than 4-methylpyrazole as an inhibitor of PNP hydroxylation (Fig. 4A). Enzyme kinetic studies were performed to analyze the type of inhibition. Lineweaver-Burk plots performed with several concentrations of SAM revealed a non-competitive type of inhibition, characterized by a decrease in V_{max} and constant K_m of about 30 μM for PNP. The K_i for SAM was around 1.5 mM (Fig. 4B). Several control experiments were performed: PNP hydroxylation required microsomes, NADPH, and PNP (Table 2). Replacing PNP with 2 mM SAM did not produce any compound with the absorbance characteristics of 4-nitrocatechol (Table 2). 2 mM SAM added after incubating microsomes with PNP plus NADPH failed to show any inhibition of enzymatic activity. Washed microsomes (using the same procedure as the one described for binding spectra) did not show significant inhibition (when compared to control microsomes subjected to the same washing procedure). MTA, SAH and methionine, when used at a 2 mM concentration, did not produce significant inhibition of PNP hydroxylation activity (Table 2).

Oxidation of ethanol was significantly inhibited by SAM with an IC_{50} of around 5 mM. 4-methylpyrazole was used as a positive control (Fig. 5A). Under these conditions, ethanol oxidation is largely P450-dependent. The contribution of catalase and/or hydroxyl radical to the oxidation of ethanol would be expected to be negligible, since the assay was performed in the presence of azide, a catalase inhibitor, and desferrioxamine, an inhibitor of microsomal hydroxyl radical formation [38]. At 5 mM, SAH, MTA and methionine did not produce any significant inhibition of ethanol oxidation (Fig. 5A).

NDMA at low concentrations is a relatively specific substrate for CYP2E1 (low K_m NDMA demethylation activity) [39]. SAM inhibited NDMA demethylation with an IC_{50} of around 5 mM. At 4 mM, 4-methylpyrazole completely inhibited NDMA oxidation (Fig. 5B).

3.5. Inhibition in microsomes expressing human cytochrome P450s

Microsomes derived from baculovirus infected insect cells engineered to express human CYP2E1 or CYP3A4 cDNA, were used for these experiments. CYP3A4 was evaluated since it is the most abundant P450 in human liver, metabolizes many important drugs, and can also activate hepatotoxins such as ethanol and acetaminophen. The IC_{50} determination for inhibition of CYP2E1 activity was performed with a single substrate (7-MFC) concentration near the apparent K_m, and multiple concentrations of SAM or

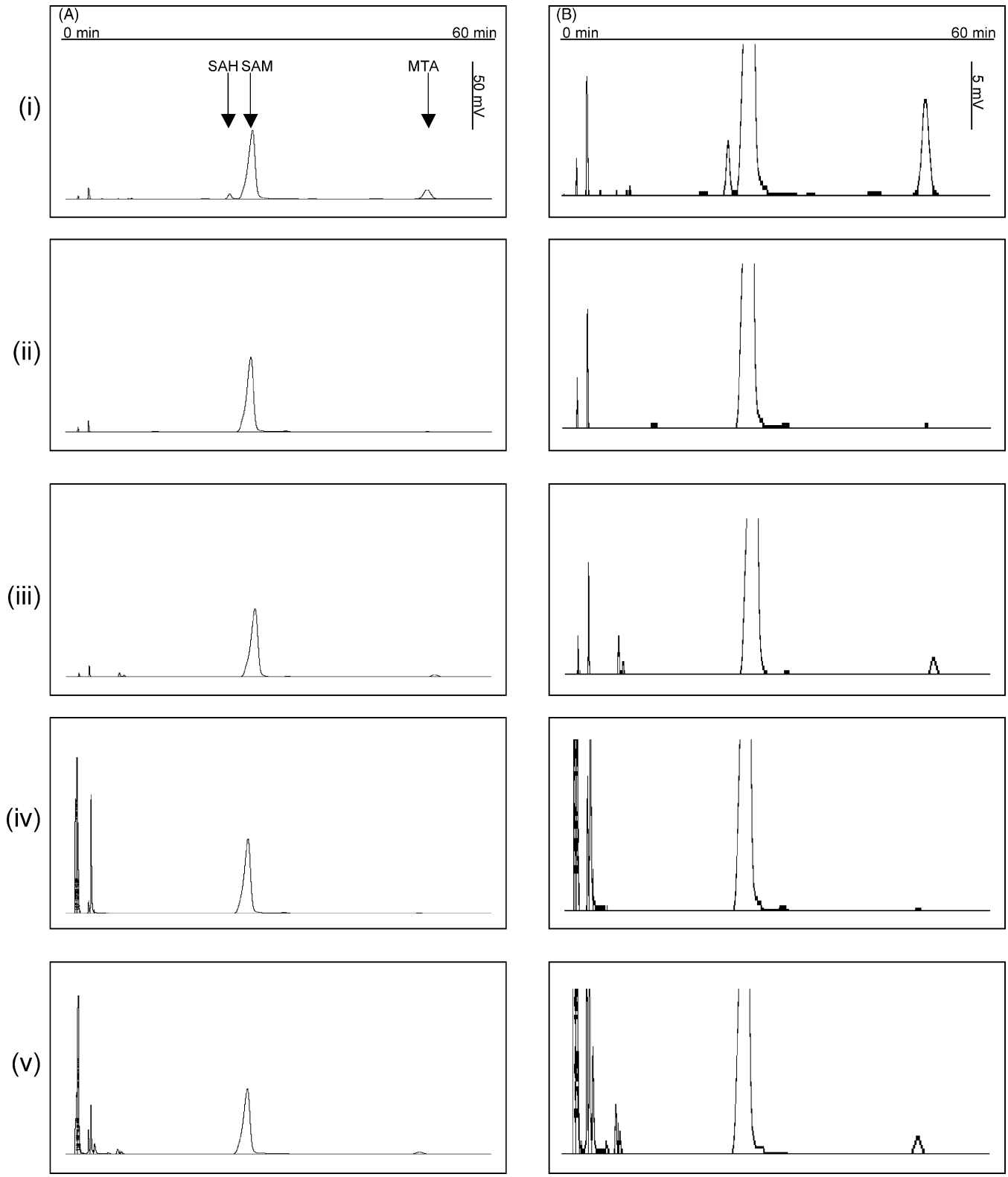

Fig. 2. Representative HPLC chromatograms of: (i) SAM (commercially available from Sigma); (ii) SAM purified according to the procedure described in Materials and Methods; (iii) Purified SAM incubated for 60 min at 37 °C; (iv) Initial incubation (time = 0 min) of a mixture containing SAM + microsomes + 1 mM NADPH; (v) 60 min incubation of the mixture containing SAM + microsomes + 1 mM NADPH. Retention times for SAM, MTA and SAH are 27, 51 and 23 min, respectively, under these conditions. Column A corresponds to the original chromatograms, showing all the peaks in-scale; the scale for column A is shown in the upper panel. Column B corresponds to reprocessed chromatograms where the sensitivity of the scale was increased in order to show the MTA and SAH peaks; the scale for column B is shown in the upper panel.

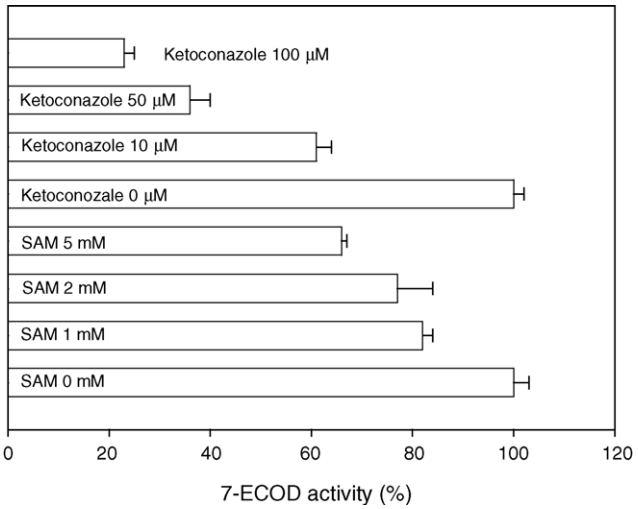

Fig. 3. Effect of SAM on 7-ECOD activity in acetone-treated rat liver microsomes. 7-ECOD activity was measured as described in Section 2, in the presence of SAM (0–5 mM) or ketoconazole (0–100 μM) (positive control). The 100% activity refers to 3.0 nmol/min/mg microsomal protein.

Table 2
Effect of SAM and SAM analogs on PNP hydroxylation

Condition	PNP activity (%)
Complete system (CS)	100 ± 3
CS + 2 mM SAM	42 ± 5
CS + 2 mM SAM added after incubation	87 ± 2
CS-microsomes	n.d.
CS-PNP	n.d.
CS-PNP + 2 mM SAM	n.d.
CS-NADPH	n.d.
CS using washed microsomes	83 ± 3
CS + 2 mM SAH	95 ± 8
CS + 2 mM MTA	85 ± 8
CS + 2 mM Methionine	90 ± 8

Control PNP hydroxylation activity was 2.0 nmol/min/mg microsomal protein; n.d. non detectable. The complete system (CS) consisted of 1 mg/mL microsomes from acetone-treated rat liver, 1 mM NADPH and 100 μM PNP, in 100 mM phosphate buffer pH 7.4, and was incubated for 15 min at 37 °C. SAM was added before (CS + 2 mM SAM), or after (CS + 2 mM SAM added after incubation) the incubation period. Controls were run either omitting microsomes, or PNP or NADPH from the complete system. The complete system was also incubated with the indicated SAM metabolites. For the control using washed microsomes, microsomes were preincubated 15 min at 37 °C in the presence of 2 mM SAM, centrifuged, resuspended in phosphate buffer, and then PNP activity was assessed (CS using washed microsomes).

diethyldithiocarbamate (DDTC) (positive control). The IC_{50} value was around 5 mM for SAM, and 6 μM for DDTC (Fig. 6A). CYP3A4 activity as evaluated by DBF oxidation showed low inhibition by SAM compared to CYP2E1; ketoconazole was used as a positive control (IC_{50} = 0.5 μM) (Fig. 6B). SAM did not interfere with the fluorometric assays, since 3 or 5 mM SAM did not alter the calibration curve performed with 7-HFC or with fluorescein under the same conditions as in the assay (data not shown). BD Biosciences cautions that the human CYP2E1 is especially highly sensitive to inhibition by ethanol, which was validated in our experiments. This necessitated purification of SAM as described above to remove the contaminating ethanol plus methanol, which are present at levels which are quite effective in inhibiting human (but not rat) CYP2E1. After purification, the remaining ethanol (about 0.01 mM) did not inhibit CYP2E1; controls with ethanol were always carried out to be compared to the effects of SAM.

3.6. Effect of SAM on microsomal production of HER

Previous studies supported the usefulness of POBN plus ethanol for kinetic studies on the production of $^{\bullet}OH$ by microsomes [29]. In the presence of iron, microsomes + NAD(P)H can produce $^{\bullet}OH$-like species which can oxidize ethanol to HER; the HER is subsequently trapped

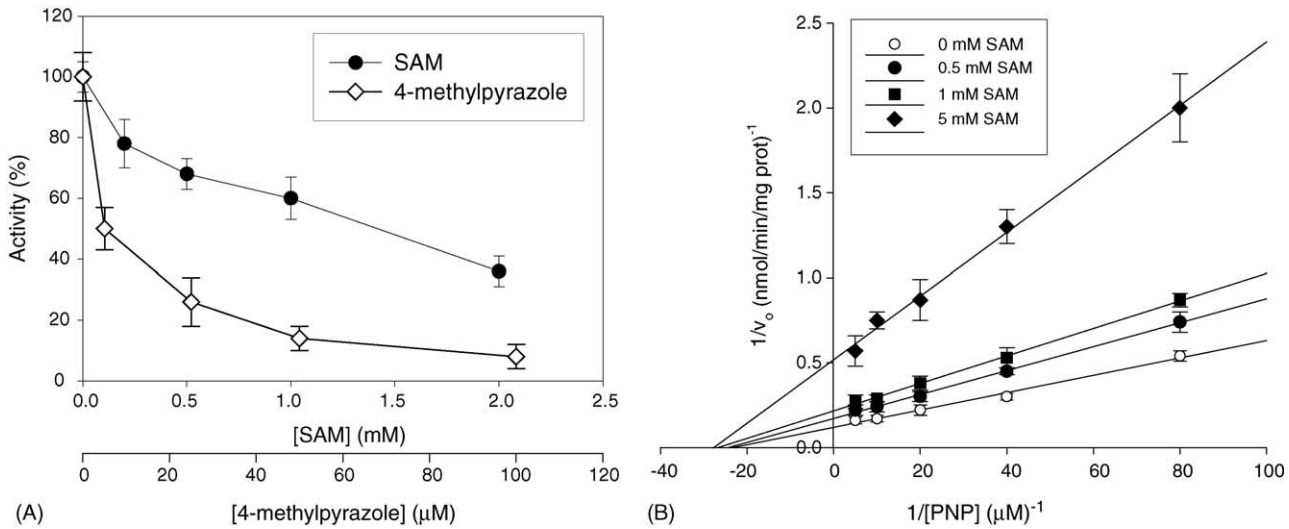

Fig. 4. Effect of SAM on PNP hydroxylation in rat liver microsomes. (A) IC_{50} determination for SAM in comparison with 4-methylpyrazole on PNP metabolism in acetone-induced rat liver microsomes; (B) Lineweaver–Burk plots of PNP metabolism in acetone-induced rat liver microsomes, in the presence of SAM (0–5 mM). The 100% activity refers to 2.0 nmol/min/mg microsomal protein.

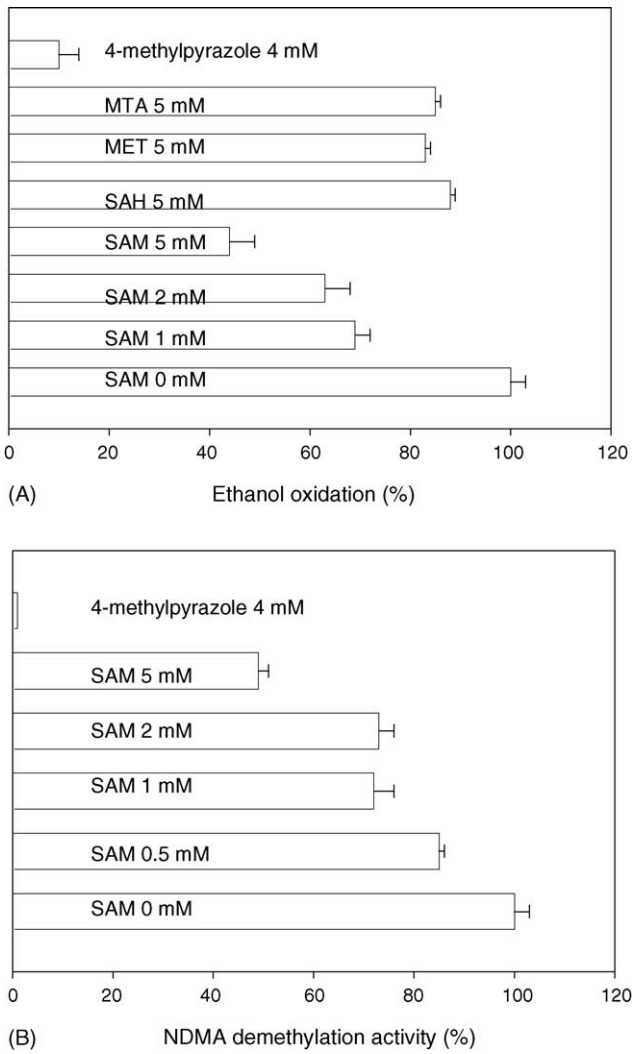

Fig. 5. (A) Efect of SAM on ethanol oxidation in acetone-treated rat liver microsomes. Ethanol oxidation was measured as described in Materials and Methods, in the presence of SAM (0–5 mM), 4-methylpyrazole (4 mM) (positive control), or metabolites of SAM at 5 mM. Control activity (100%) was 9.9 nmol/min/mg microsomal protein. (B) Effect of SAM on NDMA oxidation in acetone-induced microsomes. Control activity (100%) was 14.2 nmol/min/mg microsomal protein.

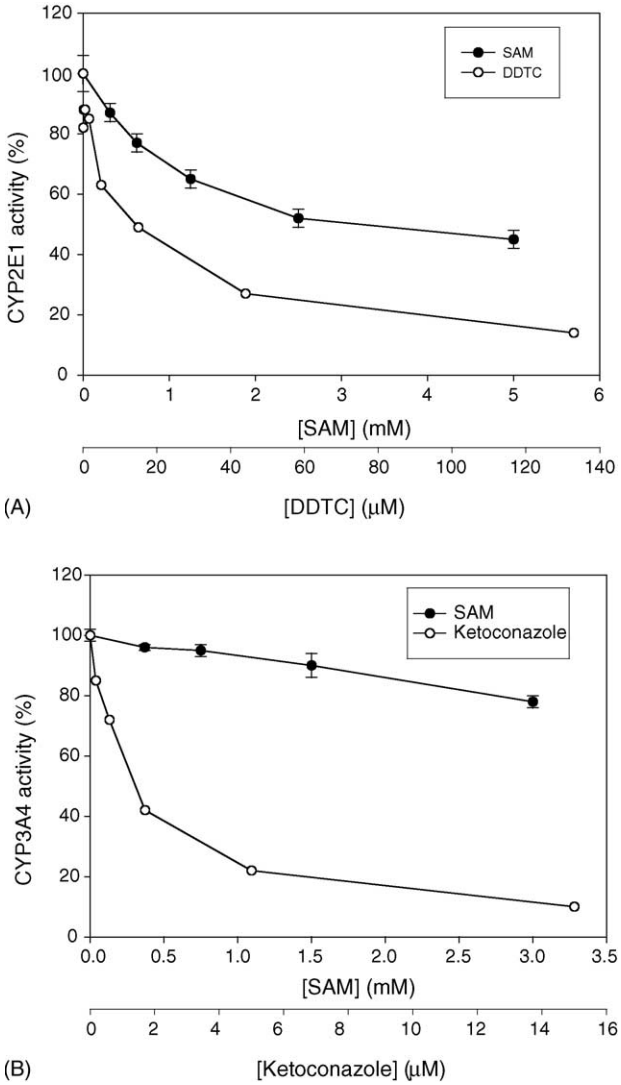

Fig. 6. Effect of SAM on P450 activity in microsomes expressing human CYP2E1 or CYP3A4. (A) IC_{50} determination for SAM in comparison with DDTC on 7-MFC metabolism in microsomes expressing human CYP2E1; (B) effect of SAM and ketoconazole on the metabolism of DBF by microsomes expressing human CYP3A4.

with POBN to produce a well characterized EPR spectrum (Fig. 7(i)). In the presence of sodium azide (a catalase inhibitor), POBN-HER formation increased dramatically (Fig. 7(ii)), while catalase inhibited the formation of HER (Fig. 7(iii)), suggesting that HER formation is quite sensitive to changes in hydrogen peroxide availability [40]. Desferrioxamine (Fe^{3+} chelator) and α,α'-dipyridyl (Fe^{2+} chelator) inhibited the generation of HER, demonstrating a role for transition metals (Fig. 7(iv) and (v)). Up to 5 mM SAM did not affect the microsomal generation of HER, in the absence or in the presence of sodium azide (Fig. 7(vi) and (vii)).

3.7. Effect of SAM on CYP2E1 activity in HepG2 cells

7-MFC is metabolized to 7-HFC (fluorescent compound, 409/530 nm excitation/emission) by CYP2E1, CYP2B6 and CYP2C8 [31]. E47 cells in the presence of 7-MFC, generated the fluorescent product in a time-dependent fashion (Fig. 8A). HepG2 cells transfected with the empty plasmid (C34 cells) did not produce any time-dependent fluorescence (Fig. 8A). In E47 cells, the generation of 7-HFC was completely inhibited by DMSO at a concentration (211 mM) known to inhibit CYP2E1 catalytic activity [41]. To evaluate the effect of SAM on CYP2E1 activity in situ, SAM (0–5 mM) was preincubated for 0–6 h prior to the addition of the fluorogenic substrate, which was further incubated for 2 h. SAM produced a concentration-dependent inhibition of the generation of 7-HFC, which was similar for initial addition of SAM (no preincubation) or if SAM was preincubated for 3 or 6 h with the E47 cells prior to the addition of the substrate (Fig. 8B). This suggests that despite its charge, sufficient

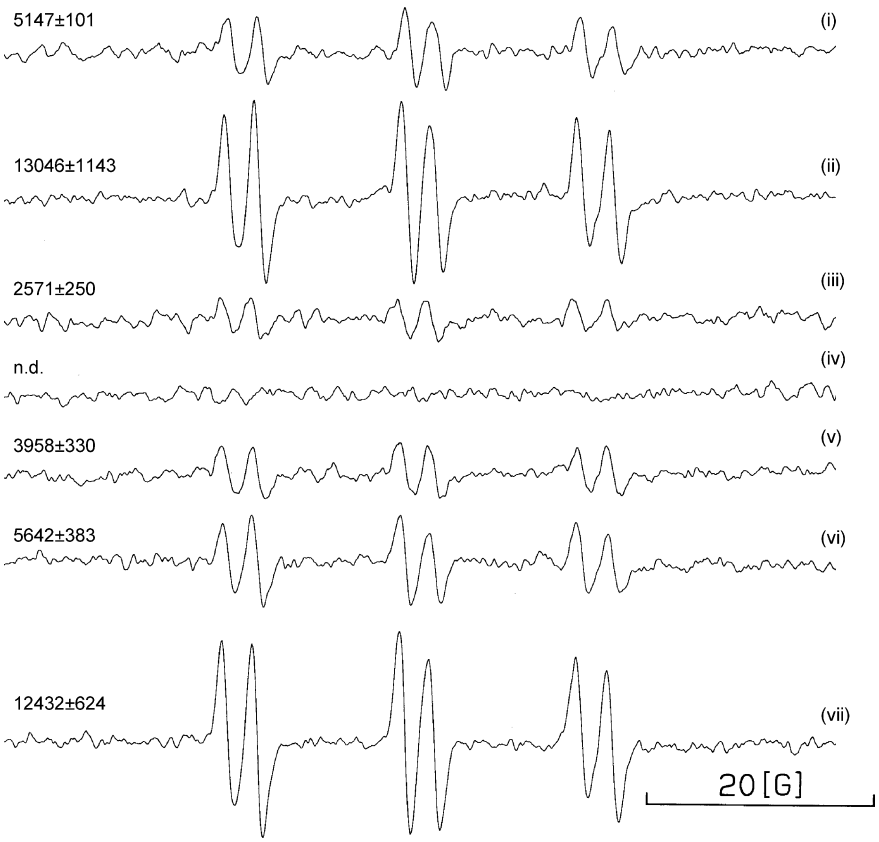

Fig. 7. NADPH-dependent generation of •OH-like species in acetone-treated rat liver microsomes. Representative EPR spectra. (i) The basic system was composed of 0.1 M phosphate buffer, pH 7.4, microsomes (0.3 mg protein/mL), 30 mM POBN, 20 mM ethanol, and 1 mM NADPH; (ii) Basic system + 1 mM azide; (iii) basic system + 100 U/mL catalase; (iv) basic system + 100 μM desferrioxamime; (v) basic system + 100 μM α,α' dipyridyl; (vi) basic system + 5 mM SAM; (vii) Basic system + 5 mM SAM + 1 mM azide. Numbers on the left side of the spectra represent the peak height of the central doublet (in arbitrary units).

SAM reaches CYP2E1 present in the endoplasmic reticulum of the E47 cells. Toxicity of SAM as evaluated by the MTT reduction assay in E47 cells, in the conditions employed did not exceed 20% (data not shown). SAM at 5 mM did not interfere with a calibration curve of 7-HFC performed in the same conditions as in the cellular assays (data not shown).

4. Discussion

Our results suggest that in in vitro models, SAM at pharmacological concentrations interacts with cytochrome P450s, in particular with CYP2E1: (a) SAM exhibited a type II binding spectra with acetone- and isoniazid-induced rat liver microsomes, and with insect microsomes engineered to express human CYP2E1, but not with control rat liver microsomes or with insect microsomes which do not express any P450, or engineered to express CYP3A4; (b) SAM inhibited a general P450-dependent reaction, the *O*-deethylation of 7-ethoxycoumarin in acetone-treated rat liver microsomes, with a $IC_{50} > 5$ mM; (c) SAM inhibited CYP2E1 activity in acetone-induced rat liver microsomes, assessed as PNP hydroxylation, ethanol and NDMA oxidation, with a IC_{50} of 1.5–5 mM; (d) SAM inhibited the activity of CYP2E1 in microsomes and in HepG2 cells engineered to only express human CYP2E1 (*O*-demethylation of 7-MFC).

Substrate binding spectra may reflect actual binding of the substrate to P450, or changes which perturb the environment surrounding the heme. Substrates which are strong ligands for iron often produce a type II difference spectrum [42], and cause spin state changes which are accompanied by a change in the redox potential of the P450, which makes P450 reduction (by NADPH P450 reductase) more difficult [42]. SAM has multiple potential coordinating atoms, including the amino and carboxylate groups, the ribose hydroxyls, and the sulfonium center [43], that may interact with iron in cytochrome P450, and/or perturb the environment around the heme. The fact that SAH, MTA or methionine do not produce an analogous binding spectrum in microsomes as does SAM, strongly suggests that the sulfonium center (missing in SAH) may be especially important for this apparent binding or perturbation of the heme environment, since SAH possesses the amino and carboxylate groups and the ribose hydroxyls. The K_s for SAM obtained through Hanes plots (Fig. 1) was 0.6 mM, and a significant binding spectra was measured

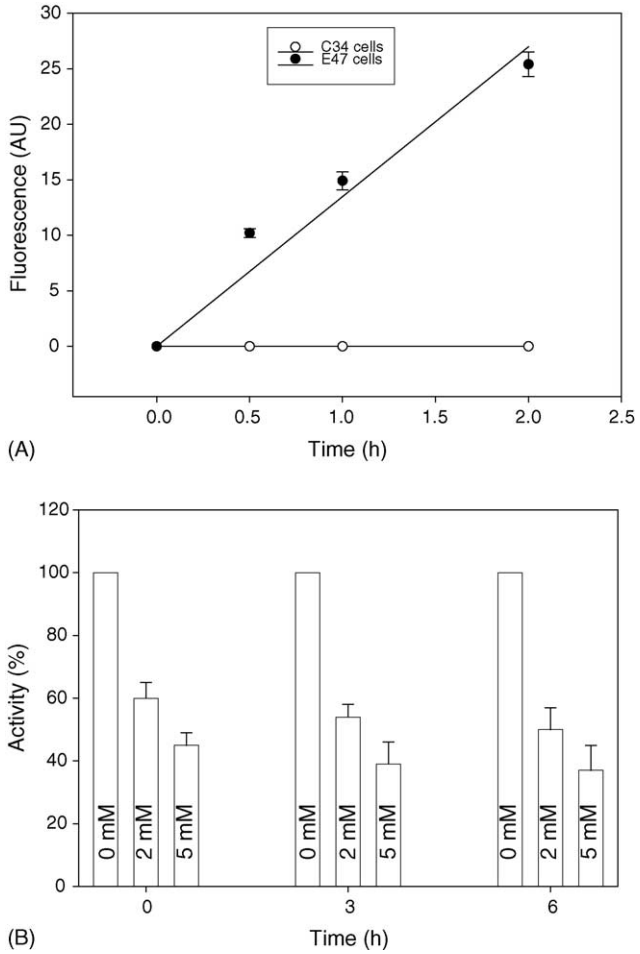

Fig. 8. Determination of CYP2E1 activity in situ. (A) HepG2 cells transfected with human CYP2E1 cDNA (E47 cells, black circles), or HepG2 cells transfected with the empty plasmid (C34 cells, white circles) were incubated for variable periods in the presence of 5 μM 7-MFC. The fluorescence of the cellular suspension was determined at 409/530 nm. (B) E47 cells were preincubated for 0, 3 or 6 h in the presence of SAM (0, 2 or 5 mM), followed by a 2 h incubation with 5 μM 7-MFC. The fluorescence of the cellular suspensions was measured at 409/530 nm.

at 100 μM SAM (Fig. 1). Intracellular concentrations of SAM are variable; reported physiological normal values are 20–100 μM. Thus, the concentrations of SAM that produce a significant binding in microsomal systems in vitro represent pharmacological and not physiological concentrations. Further studies will evaluate the in vivo binding of SAM to microsomal cytochrome P450s and the effect of multiple or chronic administration of SAM on P450 levels and activities.

SAM inhibits the in vitro activities of 7-ECOD, PNP hydroxylation, ethanol and NDMA oxidation, and 7-MFC demethylation. Several possibilities may explain the inhibitory effects of SAM: (i) SAM may act as an artificial electron acceptor and compete with P450 (or cytochrome c) for electrons from NADPH via NADPH-cytochrome P450 reductase; (ii) SAM might inhibit the catalytic activity by binding to the substrate binding site of P450 i.e., compete with substrate for binding to P450; (iii) the decreased catalytic activity may be a result of increased enzyme destruction or denaturation associated with SAM. Experiments were designed in order to test these possibilities: SAM did not inhibit the activity of NADPH cytochrome c reductase, it showed a non-competitive rather than competitive type of inhibition in kinetic studies, and did not produce significant destruction of spectrally detectable cytochrome P450, or increase the concentration of cytochrome P420, suggesting that the mechanisms proposed are not involved in the inhibitory effects of SAM. A mechanism-based type of inhibition was also ruled out, as SAM was not metabolized in the presence of microsomes plus NADPH. Moroever, inhibition by SAM was reversed by washing the microsomes. The results suggest that SAM inhibits the catalytic activity of CYP2E1 in a reversible and non competitive manner. While CYP2E1 catalytic activity was inhibited by SAM, SAM had no effect on the NADPH-dependent microsomal generation of $^{\bullet}OH$-like species. Considering that the primary role of cytochromes P450 in the generation of HER in the model system used is probably oxygen activation, and not HER formation at the active site of P450 [40], these results suggest that SAM may not interfere with oxygen activation by P450. The latter is under further study. We recently reported [44] that SAM can chelate Fe^{2+} and block the interaction of Fe^{2+} with molecular O_2 and the subsequent production of superoxide and H_2O_2. However, SAM competed poorly with H_2O_2 for interaction with Fe^{2+} [44]. Since HER formation is dependent on H_2O_2 production (inhibition by catalase), this probably explains why SAM did not inhibit HER production via a Fe^{2+} chelation mechanism. Methionine, SAH and MTA did not inhibit PNP hydroxylation or ethanol oxidation; considering that these analogs of SAM did not produce a binding spectrum with acetone-induced microsomes, these results suggest that the generation of a binding spectrum by SAM, and modulation of the heme environment, may be associated with inhibition of CYP2E1 activity. In view of the relatively high K_i (1.5–5 mM) for SAM as an inhibitor of CYP2E1 catalytic activity, the inhibition of CYP2E1 catalytic activity is not likely to be a major mechanism by which SAM protects against the CYP2E1-dependent toxicity of acetaminophen, thioacetamide, CCl_4, and ethanol. It is likely that other effects of SAM e.g. GSH precursor, membrane alterations, as discussed elsewhere [1,2,6], play a more important role in the mechanisms responsible for SAM's beneficial effects.

Since 7-ECOD activity is catalyzed by several P450s, we speculate that the higher IC_{50} by SAM may be due to a relatively weaker inhibitory effectiveness of SAM against these P450s as compared to inhibition of CYP2E1 activity as reflected by the oxidations of PNP, ethanol, NDMA, and 7-MFC. This is also suggested by the fact that control rat liver microsomes, and insect microsomes engineered to express human CYP3A4, did not develop a significant binding spectrum with SAM, and that the metabolism of

DBF in CYP3A4-expressing microsomes was less effectively inhibited by SAM compared to the metabolism of 7-MFC in CYP2E1-expressing microsomes. Other forms of P450 have not been evaluated.

At 2–5 mM, SAM inhibited CYP2E1 activity in situ in E47 cells. The mechanism involved may be related to the non-competitive inhibition of CYP2E1 catalyzed substrate oxidation by SAM found with isolated microsomes. In summary, these results indicate that in in vitro models, SAM at relatively high concentration interacts with cytochrome P450, in particular with CYP2E1. However, considering the millimolar range of concentration involved in the interaction, SAM is a considerably weaker inhibitor than other typical CYP2E1 inhibitors such as DDTC or 4-methylpyrazole.

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