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In vitro Covalent Binding of SC-42867, PGE2 Antagonist, to Rat Liver Microsomal Proteins

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Covalent binding of the reactive metabolites of SC-42867 to microsomal proteins has been examined. In the absence of an inhibitor of cytochrome oxydase (α-naphtyl-isothiocyanate) or a radical scavenger (3-terthiobutyl-4-hydroxyanisol), up to 4.0% of the total radioactivity used in the assay could irreversibly bind to proteins. In the presence of an inhibitor, the highest percentage of covalent binding observed is 0.7%, a significant decrease of the metabolism of SC-42867 was observed. These results suggest that a cytochrome P-450 dependent generation of SC-42867 metabolites significantly take part in the covalent binding process.

Key words : PGE_2, Covalent binding, Liver microsomes, Cytochrom P-450

INTRODUCTION

Covalent binding of the reactive metabolites of compounds to hepatic macromolecules appear to be the first step in the development of the hepatic lesion (Jollow *et al.*, 1973).

SC-42867 is a PGE_2 antagonist developed as potential non-narcotic analgesics for mild treatment and it possesses an 8-chlorobenzoxazepine moiety. In the previous study (Lee *et al.*, 1994), we observed that this compound was metabolized on the tricyclic moiety only. Oxidative N-dealkylation with opening of the oxazepine ring was the major metabolite obtained in rat cultured hapatocytes. Studies that have been reported in the literature on the *in vitro* bioactivation with rat liver microsomes have shown that some drugs that undergo aromatic hydroxylation such as benzen, phenytoin, mianserin and sorbinil give reactive intermediates which covalently bind to proteins. It has shown that after a single i.v. dose of [^{14}C]-SC-42867 to the beagle dog at 10 mg/kg as a solution in polyethyleneglycol, the concentration of total radioactivity and parent compound decreased rapidly, the elimination half-life of the parent compound was 2.4 hours. Nevertheless, a low level of blood and plasma concentrations of total radioactivity were maintained for a long period. These observation suggested that metabolites of [^{14}C]-SC-42867 could irreversibly bind to plasma proteins.

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In vitro irreversible binding of [^{14}C]-SC-42867 with rat liver microsomes was investigated. The study was performed in parallel with [^{3}H]-mianserin, which covalent binding is well described (Riley *et al.*, 1988) and which was used here as a positive control substance.

MATERIALS AND METHODS

Chemicals and reagents

[^{14}C]-SC-42867 (specific activity: 11.25 mCi/mmole purity: 98.4% as measured by HPLRC; HPLC with radioactivity monitor) and [N-methyl-^{3}H]-mianserin HCl (specific activity: 80 Ci/mmole) were purchased from IRE (Fleurus, Belgium). NADP, glucose-6-phosphate dehydrogenase and $MgCl_2$ were obtained from Sigma (St. Louis. Mo). ANIT and BHA were purchased from Aldrich (St. Paul, Milwaukee).

Microsome preparation

Male rats (Sprague-Dawley) were sacrificed with ether. Liver removed and washed with ice-cold HCl 0. 15 M. The pooled livers were homogenised in SET Buffer (0.25 M Sucrose, 5.4 mM EDTA, 20 mM Tris HCl, pH 7.4) as a ratio 1/4 (w/v) using a potter. The homogenate was centrifuged for 10 min at 1500 g at 4°C . The supernatant was decanted and centrifuged for 10 min at 15000 g at 4°C . The pellet was removed and the supernatant was centrifuged at 100, 000 g for 60 min at 4°C . The supernatant was decanted and the microsomal pellet was resuspended in 0.1 M sodium phosphate buffer pH 7.4 and stored at -

70°C until use. The protein concentration was measured using the Bio-rad protein assay using bovine serum albumin as a standard.

Incubation with rat liver microsomes

All incubations were performed at 37°C in shaking water bath by mixing approximately 5 mg of microsomal proteins (2 ml of the microsomal preparation described above), NADP (1 µM), $MgCl_2$ (15 µM), glucose-6-phosphate dehydrogenase (2 Eu) and a test article at various concentrations. Incubations were made using [^{14}C]-SC-42867 (2.5, 5, 10 and 25 µM) or [^{3}H]-mianserin (10 µM). Prior to addition of the test sample, the microsomal preparation was preincubated for 5 minutes at 37°C with or without one of the following inhibitors: (α-naphthylisothiocyanate (ANIT), an inhibitor of cytochrome oxidase (500 µM) and 3-terthiobutyl-4-hydroxyanisol (BHA), a free radical scavenger (500 µM).

And the sample were incubated for another 2 hours at 37°C, 6 ml methanol was added to terminate the enzymatic reaction. Microsomes boiled at 100°C for 5 min were used for the measure of the irreversible binding background.

Measure of the irreversible binding of radiolabelled material

After addition of the methanol, the mixture was centrifuged at 10,000 g for 10 min. The supernatant was removed and the pellets were exhaustively extracted with methanol. The liquid phases from each extraction were collected and used for further HPLRC analysis.

The protein pellets were resuspended in water and aliquotes were taken for liquid scintillation counting (LSC).

HPLRC analysis

200 µl aliquots of the supernatant obtained above were used for LSC measurements. The remainder of the supernatant was evaporated to dryness. The residue was dissolved in 500 µl methanol and centrifuged at 9,000 g for 30 min. This procedure was repeated twice. The supernatant was filtered (Miller-HV, 0.45 µM, Millipore, France) and analysed by HPLRC. The labelled compound and its metabolites from the incubation were analysed by reverse-phase HPLC. The chromatographic system consisted of a Waters 5600 pump and a Berthld HPLC radioactivity monitor LB 506D equipped with an yttrium glass cell.

The mobile phase was eluted successively through a semi-preparation Waters ^{18}C-µ-bondapack column (300 mm × 7.8 mm) and an analytical Waters ^{18}C-µ-bondapack column (300 mm × 3.9 mm). The flow rate was 1.0 ml/min. The mobile phase was made of water and methanol and the following conditions were used: The column was equilibrated for 5 min with water/methanol 35/65 (v/v), After injection of the sample, the amount of methanol was raised to 70% (v/v) using a linear gradient for 2 minutes. The percentage of methanol was then immediately increased up to 75% (v/v) and maintained at that concentration for 20 min. Finally a linear gradient raised the methanol concentration to 100% in 5 min.

RESULTS AND DISCUSSION

The data on an irreversible covalent binding of [^{14}C]-SC-42867 to proteins was shown in Fig. 1 (Table 1). The percentage of compound covalently bound is concentration dependent and reaches a maximum of 4.0% of the total radioactivity at the lowest concentration of 2.5 µM.

The part taken by the metabolites in that process is demonstrated by the significant reduction of covalent binding observed at every concentration when a cytochrome oxidase inhibitor (ANIT) or a radical scavenger (BHA) is added to the incubation mixture. Table 1 and Fig. 1 show that the level of covalent binding only reach 0.68% or 0.63% of the total radioactivity at the lowest concentration, after addition of ANIT or BHA, respectively.

Metabolism profiles measured by HPLRC confirm the results described above. Fig. 2 shows the profiles obtained without the presence of inhibitor (2a), in the presence of ANIT (2b) and BHA (2c) as well as the profile of an injection of a standard sample of [^{14}C]-SC-42867 (2d) indicating the retention time of the parent compound.

The structure of metabolites I, II were determined in the course of the previous "*in vitro*" experiments us-

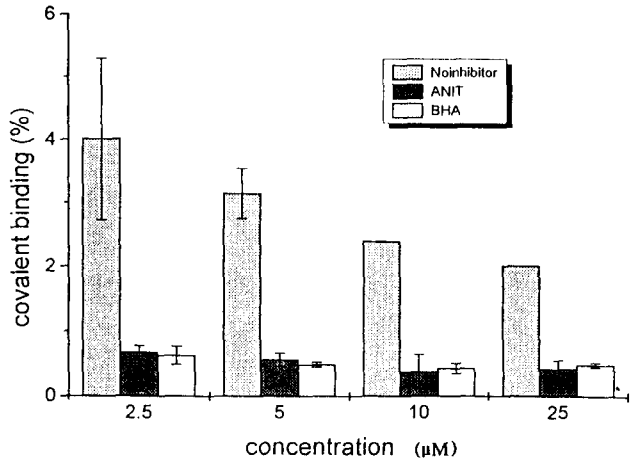

Fig. 1. *In vitro* irreversible binging of ^{14}C-42867 to proteins after 2 hours of bioactivation with rat liver microsomes

ing cultured rat hapatocytes (Lee *et al.*, 1994) and the structure of those are displayed in Fig. 3.

Table 1. *In vitro* irreversible binding of ^{14}C-42867 to proteins after 2 hours of bioactivation with rat liver microsomes

	Experiment 1	Experiment 2	Mean	SD
[^{14}C]-SC-42867 25 μM				
No inhibitor	2.03	2.03	2.03	0.00
ANIT	0.52	0.33	0.43	0.13
BHA	0.52	0.46	0.49	0.04
[^{14}C]-SC-42867 10 μM				
No inhibitor	2.40	2.39	2.40	0.01
ANIT	0.58	0.20	0.39	0.27
BHA	0.50	0.38	0.44	0.08
[^{14}C]-SC-42867 5 μM				
No inhibitor	2.87	3.42	3.15	0.39
ANIT	0.64	0.50	0.57	0.10
BHA	0.52	0.46	0.49	0.04
[^{14}C]-SC-42867 2.5 μM				
No inhibitor	3.10	4.91	4.01	1.28
ANIT	0.75	0.61	0.68	0.10
BHA	0.53	0.73	0.63	0.14
[^{3}H]-mianserin 10 μM				
No inhibitor	3.53	4.93	4.23	0.99
ANIT	2.26	2.00	2.13	0.18
BHA	0.89	0.77	0.83	0.08

So are at least two of the three metabolites observed formed by oxidative hydroxylation of the compound. Fig. 2b and 2c show that the metabolism is inhibited by the presence of either ANIT or BHA.

The results observed here are fully consistent with those observed in our study with mianserin (Table 1) or described in the literature for a large number of compound. Such as phenytoin (Billings *et al.*, 1986; Roy *et al.*, 1988; Riley *et al.*, 1988), benzene (Brofuehrer *et al.*, 1988), which all undergo aromatic

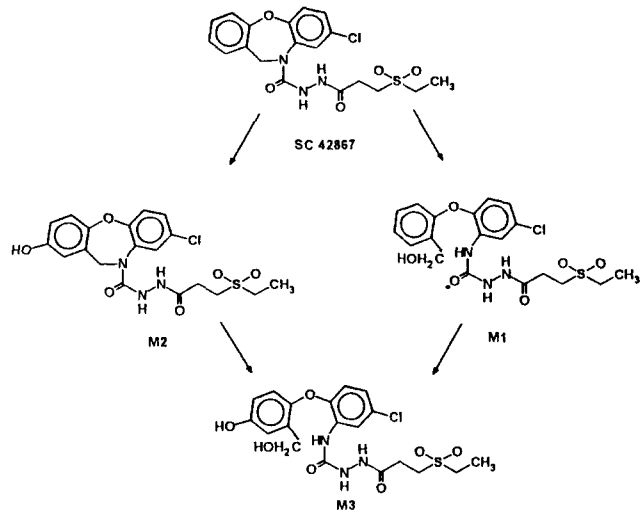

Fig. 3. Proposed metabolic pathways of SC-42867 in cultured rat hepatocytes (Lee *et al.*, 1994)

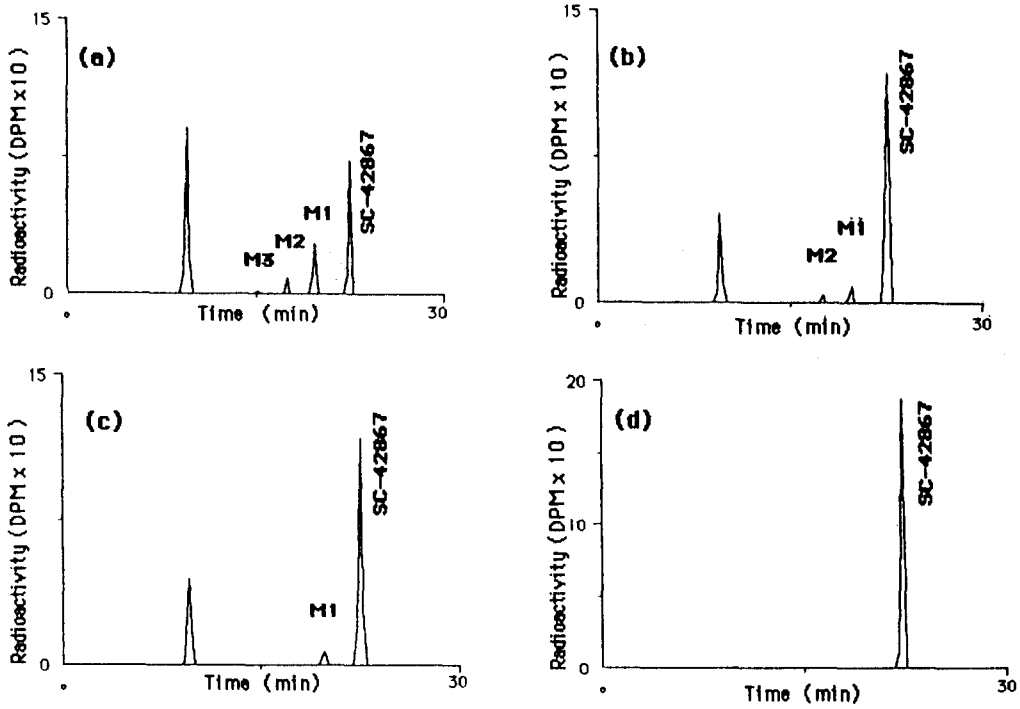

Fig. 2. HPLRC profiles of SC-42867 and its metabolites formed by incubation with rat liver microsomes for 2 hours

hydroxylation and give protein reactive metabolites.

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