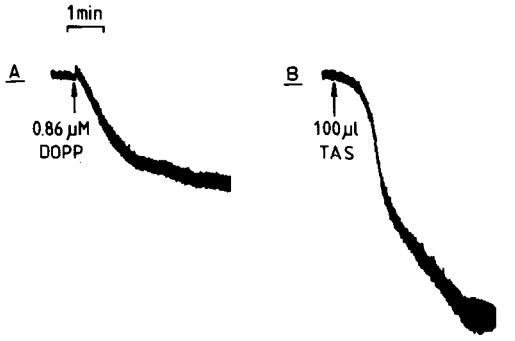

Fig. 3. A. Aggregation of human platelets previously desensitised to PAF by 0.86 μM DOPP. B. Aggregation of platelets previously desensitised to PAF by 100 μl of TAS.

plasma within 30 sec of aggregation and its production was maximal within 4 min with a half-life of 20 min. Washed human platelets which failed to respond fully to DOPP stimulation in terms of aggregation still secreted TAS into buffer solution. The aggregating activity of TAS was shown to be distinct in its properties from that of DOPP, ADP, PAF and the products of arachidonate metabolism. TAS could not be purified by conventional chromatography on silica gel as adsorbant and lost its activity as a result of freezing for 24 hr and by prior incubation with trypsin, accordingly it is probable that TAS is a polypeptide.

Acknowledgements—This work was supported by the Vandervell Foundation.

Department of Pharmacognosy, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, U.K.

MARY C. EDWARDS
ELIZABETH M. WILLIAMSON
EVELYN FORMUKONG
GAVIN BROOKS
FRED J. EVANS

REFERENCES

1. J. Westwick, E. M. Williamson and F. J. Evans, *Thrombosis Res.* **20**, 683 (1980).
2. M. C. Edwards, S. E. Taylor, E. M. Williamson and F. J. Evans, *Acta pharmac. tox.* **53**, 177 (1983).
3. M. Hergenhahn, S. Kusumoto and E. Hecker, *Experientia* **30**, 1438 (1974).
4. C. Ellis, N. Morrice, A. Aitkin, P. Parker and F. J. Evans, *J. Pharm. Pharmac.* **37** 23P (1985).

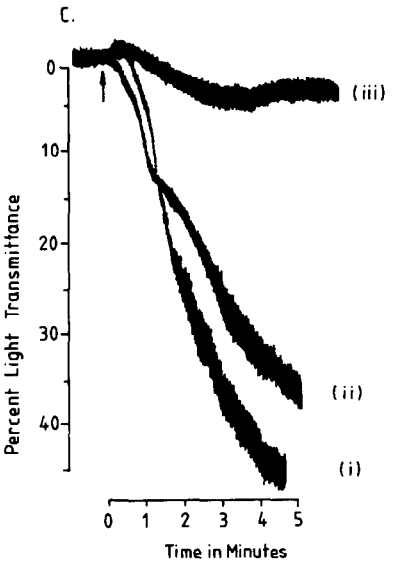

Fig. 4. (i) Aggregation of human platelets induced by 0.43 μM DOPP added to PRP 1 min following the addition of trypsin (0.1 mg/ml). (ii) Aggregation induced by TAS (100 μl) following secretion induced by DOPP (0.43 μM). (iii) The effect of 100 μl of plasma from (i) on recipient PRP.

5. T. Gilmore and G. S. Martin, *Nature, Lond.* **306**, 487 (1983).
6. M. Naka, M. Nishikawa, R. S. Adelstein and H. Hidaka, *Nature Lond.* **306**, 490 (1983).
7. M. C. Edwards and F. J. Evans, *Bot. J. Linn Soc. Proc. Anniversary Meeting of the Linnean Soc*, p 1 (1987).
8. E. M. Williamson, J. Westwick, V. V. Kakkar and F. J. Evans, *Biochem. Pharmac.* **30**, 2691 (1981).
9. F. J. Evans and R. J. Schmidt, *Acta Pharmac. tox.* **45**, 181 (1979).
10. R. A. Mufson, P. Kulkarni, K. E. Eakins and I. B. Weinstein, *Cancer Res.* **89**, 3602 (1979).
11. S. N. Khan, P. A. Lane and A. D. Smith, *Eur. J. Pharmac.* **107**, 189 (1985).
12. D. E. Agawa, L. C. McPhail, M. L. Daniel, D. A. Bass and C. E. McCall, *6th International Conference on Prostaglandins and Related Compounds,* Florence, Italy, p. 143. Raven Press, New York (1986).

Biochemical Pharmacology, Vol. 36, No. 14, pp. 2421–2424, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Metabolic activation of the tricyclic antidepressant amineptine by human liver cytochrome P-450

(*Received* 18 *November* 1986; *accepted* 26 *January* 1987)

Amineptine is widely used in several countries. Like other tricyclic antidepressants [1, 2], amineptine may produce hepatitis in some patients [3, 4]. We recently reported that amineptine is transformed by hamster liver cytochrome P-450 into a chemically reactive metabolite that may covalently bind to hepatic proteins both *in vitro* and *in vivo* [5, 6].

Cytochrome P-450 isozymes present in human liver are not exactly the same as those present in animals [7]. It was therefore of interest to see whether a similar metabolic activation also occurs with human liver microsomes.

Materials and methods

Chemicals. Amineptine hydrochloride and [11-^{14}C]-amineptine hydrochloride were generously given by Servier laboratories (Neuilly, France). The radiochemical (24 mCi·mmol^{-1}), labelled on the middle ring of the tricyclic structure, was prepared by Commissariat à l'Energie

Atomique, Gif-sur-Yvette, France. Its radiochemical purity was found to be higher than 99% by high performance liquid chromatography.

Human liver microsomes. Part of a liver specimen removed by surgical biopsy or during a hepatic tumor resection was obtained in 6 patients undergoing elective abdominal surgery for various reasons (digestive carcinomas, gall-bladder stones, hepatic tumor) and in whom a histological examination of the liver was medically required. We excluded alcoholic patients or those who had taken drugs known to induce microsomal enzymes [8] during the two weeks preceding surgery; patients taking other drugs were not excluded, inasmuch as drugs had to be taken for premedication and anaesthesia anyway. Patients were premedicated with alimemazine, atropine and hydroxyzine and were anaesthetized with droperidol, enflurane, fentanyl, thiopental sodium, and pancuronium bromide.

Part of the liver specimen was placed in Bouin's fluid, and sent to the pathologist. Another fragment of the surgical liver biopsy, or parts of the removed normal liver during tumor resection, were stored at −20° until the conclusion of the pathologist was available. Only those liver fragments with a normal liver histology were used in the present study.

The liver fragment was thawed, blotted dry, weighed and homogenized in 3 vol. of ice-cold 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 60 min. Microsomal pellets were stored at −20° until analyzed, 1–3 days later.

Animal liver microsomes. For comparison, a few studies were also made with liver microsomes from rats, mice and hamsters. Male Sprague–Dawley rats [Crl:CD®(SD)BR] weighing 180–220 g and male Swiss mice [Crl:CD®-1(ICR)BR] weighing 25–35 g were purchased from Charles River France (Saint-Aubin-les-Elbeuf, France). Male golden Syrian hamsters, weighing 90–110 g, were purchased from Fichot (Ormesson, France). Animals were fed *ad libitum* with a normal diet: Autoclavé 113, UAR, France (for rats), and M25 biscuits, Extra Labo, Provins, France (for mice and hamsters). Hepatic microsomes were prepared as described above.

Proteins and cytochrome P-450. Microsomal protein concentration was determined by the method of Lowry *et al.* [9]. Cytochrome P-450 was determined as described by Omura and Sato [10].

In vitro *covalent binding studies.* Unless otherwise indicated, the standard incubation was performed as follows; [11-^{14}C]amineptine (1 μCi/ml, 1 mM), dissolved in 10 μl of 0.1 N NaOH, was added to an incubation mixture containing: NADP (1 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate-dehydrogenase (3 enzyme units/ml), $MgCl_2$ (6 mM) and microsomes from 125 mg of liver, in a total volume of 1 ml 0.07 M KCl, 0.13 M sodium potassium phosphate buffer, pH 7.4. A zero time sample (250 μl) was removed, and the remaining mixture was incubated under air, with shaking at 37° for 5 min; another sample (250 μl) was then removed. For experiments in anaerobiosis, an oxygen-consuming system consisting of glucose (13 mM), glucose oxidase (8 U/ml) and catalase (1000 U/ml) was added in the incubation mixture; nitrogen was bubbled in the ice-cold incubation mixture for 5 min and then, during 5 min of preincubation at 37°. [11-^{14}C]amineptine was then added, and the incubation was carried out for 5 min, with nitrogen bubbling in the incubation mixture.

The amount of [11-^{14}C]amineptine metabolite irreversibly bound to proteins was measured as previously reported [5, 6]. Radioactivity in the zero time sample was subtracted from that in the incubated sample [5].

Statistical analysis. Analysis of variance and Dunnett's test were used for statistical analysis when one control group was compared to several other groups. The Student's *t*-test was used for comparisons between two groups.

Results and discussion

The present results show that a reactive amineptine metabolite is also formed with human liver microsomes. Indeed, incubation of [11-^{14}C]amineptine with human liver microsomes and an NADPH-generating system resulted in the irreversible binding of a [^{14}C]metabolite to microsomal proteins (Fig. 1). Binding to human liver microsomes

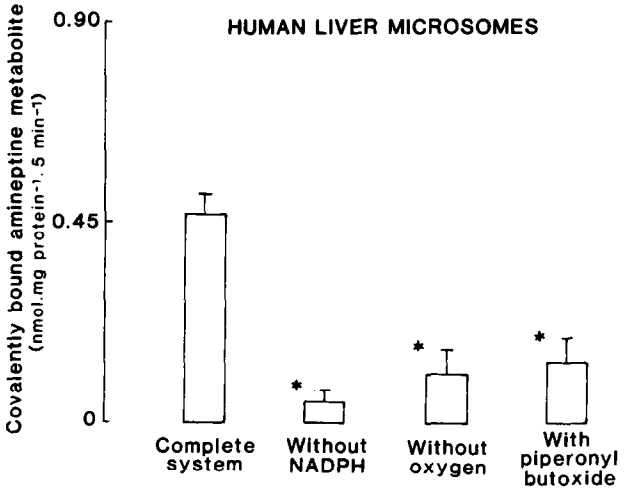

Fig. 1. Cofactor requirements and effect of a cytochrome P-450 inhibitor on *in vitro* covalent binding with human liver microsomes. In the "complete system", the incubation mixture (1 ml) contained [11-^{14}C]amineptine (1 μCi/ml, 1 mM), an NADPH-generating system and human liver microsomes from 125 mg of liver. In some flasks, the NADPH-generating system was omitted. Other flasks were incubated under nitrogen, in the presence of an oxygen depleting system (see Materials and Methods). In others, piperonyl butoxide (4 mM) was added in the incubation mixture. After 5 min of incubation, under air, at 37°, the amount of [^{14}C]metabolite irreversibly bound to microsomal proteins was determined. Results are means ± SEM for six determinations. The asterisks indicate significant differences from the value in the complete system ($P < 0.01$).

required NADPH and oxygen and was markedly inhibited by piperonyl butoxide (Fig. 1), suggesting that metabolic activation is mediated by cytochrome P-450. Similar observations have been made in hamster liver microsomes [5]. It is noteworthy, however, that the *in vitro* covalent binding of an amineptine metabolite to microsomal proteins was greater with human liver microsomes than with hamster liver microsomes (Fig. 2). The difference became quite noticeable when covalent binding was expressed per nmol of cytochrome P-450, i.e. the enzyme responsible for metabolic activation (Fig. 2). Indeed, expressed in this way, covalent binding was much greater with human liver microsomes than with microsomes from mice, hamsters or rats (Fig. 2). This may indicate that some human liver cytochrome P-450 isozyme(s) may actively form the reactive amineptine metabolite(s).

The chemical structure of the reactive metabolite(s) of amineptine remains unknown. Although 1,1,1-trichloropropene 2,3-oxide (0.1 mM) significantly increased *in vitro* covalent binding in hamster liver microsomes [5], the increase with human liver microsomes was not significant (Fig. 3). Further studies are required to delineate better the chemical structure of the reactive amineptine metabolite(s) [5].

In hamsters, physiologic concentrations of glutathione decreased *in vitro* covalent binding to undetectable levels [6]. Similarly, 4 mM glutathione almost prevented the *in vitro* covalent binding to human liver microsomes (Fig. 3), suggesting that in humans as in hamsters, the reactive metabolite is actively detoxified by glutathione [6].

Previous findings in hamsters have shown that the reactive amineptine metabolite has no direct toxicity to the liver [6]. Indeed, even in hamsters first pretreated with phenobarbital, then depleted of glutathione by pre-administration of phorone, and then injected with 100-times the human dose of amineptine (on a weight basis), there was no liver cell necrosis [6].

In humans, hepatitis after therapeutic doses of aminep-

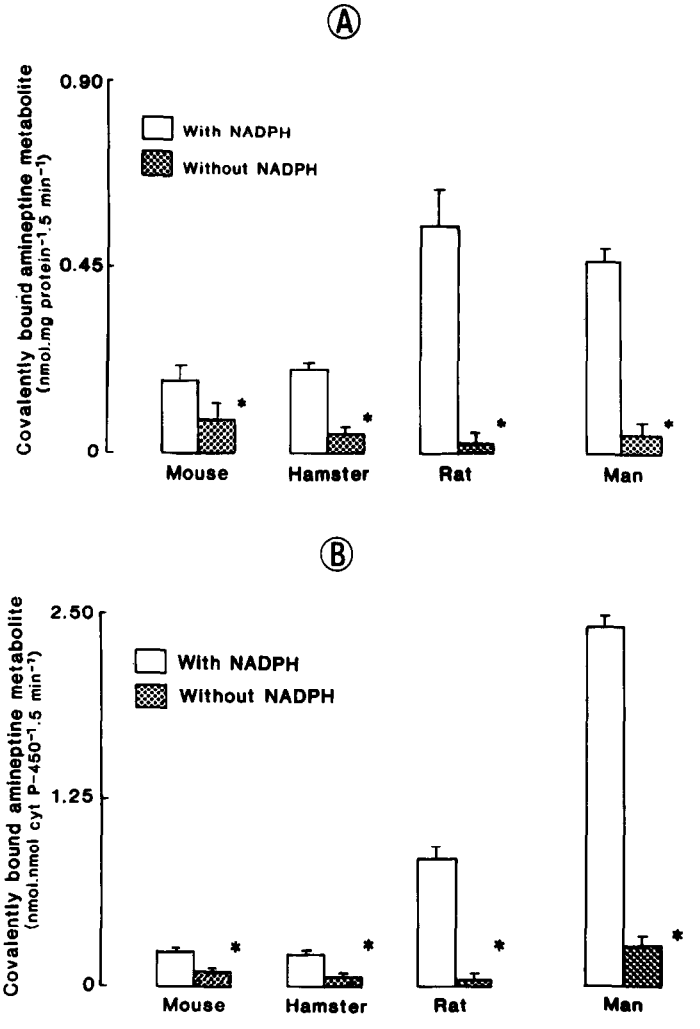

Fig. 2. Comparison of *in vitro* covalent binding with liver microsomes from mice, hamsters, rats and humans. Incubations were made as in Fig. 1, with or without an NADPH-generating system. Microsomal protein (mean ± SEM) was 34 ± 1 mg·g liver^{-1} in mice, 41 ± 2 in hamsters, 39 ± 1 in rats and 36 ± 1 in humans; microsomal cytochrome P-450 was 0.9 ± 0.2 nmol·mg microsomal protein^{-1} in mice, 1.0 ± 0.1 in hamsters, 0.7 ± 0.1 in rats and 0.19 ± 0.03 in humans. The upper figure (A) shows *in vitro* covalent binding expressed per mg of microsomal protein. The lower figure (B) shows covalent binding expressed per nmol of cytochrome P-450, i.e. the enzyme responsible for metabolic activation. Results are means ± SEM for six determinations. The asterisks indicate significant differences from the value in the incubation with NADPH ($P < 0.01$).

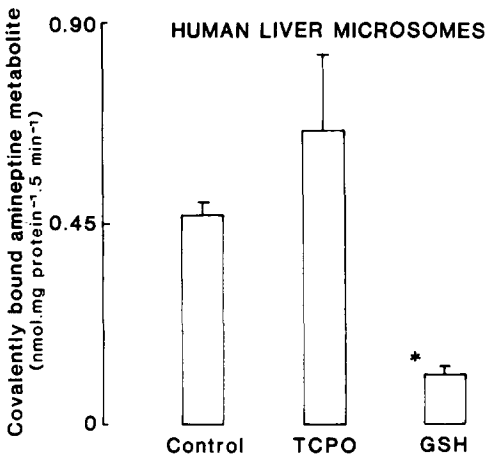

Fig. 3. Effects of glutathione and an inhibitor of epoxide hydrolase on *in vitro* covalent binding with human liver microsomes. In some flasks, 0.1 mM of 1,1,1-trichloropropene 2,3-oxide (TCPO) or 4 mM of reduced glutathione (GSH) were added to the incubation mixture. Results are means ± SEM for six determinations. The asterisk indicates a significant difference from the value in the control incubation ($P < 0.01$).

tine has the clinical features of an allergic phenomenon [3, 4]. Thus, hepatitis occurs after repeated doses, appearing 10 days to 6 months after the onset of the treatment; hepatitis is usually associated with hypersensitivity manifestations, and promptly recurs after a rechallenge. Hypothetically, the metabolite-macromolecule complexes may, in a few immunologically predisposed subjects, lead to immunization against the drug-altered macromolecules, resulting in an allergic type of hepatitis [6].

We conclude that amineptine is transformed by human cytochrome P-450 into a reactive metabolite which is efficiently detoxified by glutathione or may covalently bind to hepatic proteins.

Acknowledgements—We are much grateful to SERVIER laboratories for granting a two years-fellowship to one of us and for providing [11-^{14}C]amineptine.

Unité de Recherches de Physiopathologie Hépatique (INSERM U 24), and Clinique Chirurgicale, Hôpital Beaujon, 92118 Clichy, France

JEAN GENÈVE
DOMINIQUE LARREY
GILLES AMOUYAL
JACQUES BELGHITI
DOMINIQUE PESSAYRE

REFERENCES

1. B. H. Ch. Stricker and P. Spoelstra, *Drug-Induced Hepatic Injury. A Comprehensive Survey of the Literature on Adverse Drug Reactions up to January 1985.* Elsevier, Amsterdam (1985).
2. H. J. Zimmerman, *Hepatotoxicity. The Adverse Effects of Drugs and other Chemicals on the Liver.* Appleton-Century-Crofts, New York (1978).
3. J. Andrieu, J. Doll and C. Coffinier, *Gastroenterol. clin. biol.* **6**, 915 (1982).
4. P. Bories, G. Pomier-Layrargues, J. P. Chotard, D. Citron, D. Capron and H. Michel, *Nouv. Presse Med.* **9**, 3689 (1980).
5. J. Genève, D. Larrey, P. Lettéron, V. Descatoire, M. Tinel, G. Amouyal and D. Pessayre, *Biochem. Pharmac.* **36**, 323 (1987).
6. J. Genève, C. Degott, P. Lettéron, M. Tinel, V. Descatoire, D. Larrey, G. Amouyal and D. Pessayre, *Biochem. Pharmac.* **36**, 331 (1987).
7. P. P. Wang, P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Dadlubar, D. Larrey and F. P. Guengerich, *Biochemistry* **22**, 5375 (1983).
8. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).

Biochemical Pharmacology, Vol. 36, No. 14, pp. 2424–2427, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Microsomal cholesterol epoxide hydrolase activity in 2-acetylaminofluorene-induced rat liver hyperplastic nodules

(*Received* 25 *August* 1986; *accepted* 18 *December* 1986)

Cholesterol 5,6-oxide formed endogenously from lipid-peroxidation-mediated epoxidation of cholesterol [1] has been shown to possess mutagenic and carcinogenic properties [2]. This compound is formed in the skin of mice upon exposure to ultraviolet light [3] and also causes malignant transformation of Chinese hamster cells in culture [4]. Cholesterol epoxide hydrolase, which hydrolyzes cholesterol 5,6-oxide to cholestan 3β,5α,6β-triol, thus has an important role in the detoxification of this epoxide. This enzyme in rat and mouse liver microsomes is distinct from the well studied microsomal epoxide hydrolase (EC 3.3.2.3) that hydrolyzes several arene and alkene oxides [5, 6]. Hyperplastic nodules induced in rat liver by chronic feeding of 2-acetylaminofluorene and believed to be the precursors for the later stages of hepatocellular carcinoma contain 3- to 4-fold elevated levels of microsomal epoxide hydrolase (assayed using styrene oxide as substrate) [7]. A number of heterocyclic compounds such as harman, norharman, ellipticine, isoquinoline, metyrapone, chalcone, and 9-fluorenone have been reported to be potent stimulators of microsomal styrene oxide hydrolase activity [8, 9], and the degree of stimulation with these compounds has been found to be greater with the enzyme from hyperplastic nodules [9]. No reports are available on the level of cholesterol epoxide hydrolase in hyperplastic nodules and how the enzyme is affected by the compounds that stimulate styrene oxide hydrolase. In the present study, hyperplastic liver nodules were found to contain reduced levels of cholesterol epoxide hydrolase activity, and the various stimulators of styrene oxide hydrolase inhibited cholesterol epoxide hydrolysis by microsomes from control and hyperplastic nodules.

Materials and methods

[4-^{14}C]Cholesterol (sp. act. 54 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Harman, norharman, isoquinoline, metyrapone, 9-fluorenone, chalcone, 1,1,1-trichloropropene oxide and acetonitrile