a formation of several different adducts (about 20) whose number was hard to establish due to small differences in their R_f values. The extent of modification amounted to one adduct per about 1000 nucleotides.

In conclusion the study indicates that 1-nitro-9-aminoacridines are able to covalently bind to DNA of HeLa S_3 cells to an exceptionally high degree, from 1 molecule of the drug per 330 to 1000 nucleotides for C-857 and C-1006, respectively. As is shown in Fig. 1, the compounds studied differ in their EC_{50} values, thus, in their cytotoxic activity. The ability to covalently bind to DNA seems to be correlated with cytotoxic activities since when the HeLa S_3 cells were treated with the drugs at the concentrations constituting the same multiplicity of EC_{50} ($1360 \times EC_{50}$ in our experiments), the degree of covalent binding to DNA remained approximately the same for all the compounds. This means that more biologically active derivatives of 1-nitro-9-aminoacridine are also more potent DNA binding agents.

The position of a nitro group is very important since, as it was shown, 1-nitro derivatives are able to bind covalently to DNA while the 2-nitro isomer of Ledakrin did not give rise to any DNA adducts. As both 1- and 2-nitroacridines intercalate into DNA and the biologically inactive 2-nitro derivative does not form DNA adducts, it means that not intercalation but covalent binding to DNA is a prerequisite for biological activity of 1-nitro-9-aminoacridines. On the other hand, since all the 1-nitro derivatives studied possess the same nitro-substituted acridine core, it must be the structure of the side chain that is responsible for the degree of covalent binding to DNA. The side chain also has influence on the number of different DNA adducts that a given derivative can form (e.g. 5 DNA adducts for Ledakrin and at least 20 for C-1006). The autoradiograms presented in this paper do not allow one to ascertain whether any of the DNA adducts detected are common for all investigated compounds. It is interesting, however, that the number of DNA adducts does not correspond to cytotoxic activity.

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Acknowledgements—The authors wish to thank A. Składanowski M.Sc. and M. Bontemps-Gracz M.Sc. for help with the preparation of the manuscript. These studies were supported by National Cancer Programme Grant CPBR 11.5/115.

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Biochemical Pharmacology, Vol. 36, No. 23, pp. 4171–4173, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Studies on *N*-demethylation of methamphetamine by means of purified guinea-pig liver flavin-containing monooxygenase

(*Received* 17 *March* 1987; *accepted* 23 *June* 1987

The metabolism of methamphetamine (MP) has been studied extensively in mammals including man [1]. *N*-Demethylation, one of the major metabolic pathways of MP, has been recognized to proceed via at least two independent routes. One is the *C*-hydroxylation pathway catalyzed mainly by cytochrome (cyt.) P-450 [2] and another is *N*-hydroxylation pathway catalyzed mainly by flavin-containing monooxygenase (FMO) [3]. Our previous work [4] revealed that *N*-demethylation of MP proceeds mainly by the *N*-hydroxylation pathway in guinea pigs. In the present study, the role of FMO in *N*-hydroxylation of MP and *N*-demethylation of *N*-hydroxy-MP was investigated using liver microsomes and purified FMO from guinea-pig liver microsomes.

Materials and methods

Chemicals. d-MP hydrochloride was purchased from Dainippon Pharmaceutical Co., Osaka. Neutral oxalates of *N*-hydroxy-MP and *N*-hydroxyamphetamine (*N*-hydroxy-AP) were synthesized by the method of Coutts *et al.* [5]. All other reagents used were from the sources described elsewhere [4, 6] or of the highest quality commercially available.

Purification of FMO. Liver microsomes of Hartley guinea pigs (250–350 g) prepared by the method described earlier [4] were solubilized with 1.0% Emulgen 911 in Buffer A (10 mM potassium phosphate (pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1.0 mM EDTA and 20% glycerol). Solubilized supernatants obtained by ultracentrifugation at 105,000 *g* for 60 min were diluted with 4 vol. of Buffer A, and applied onto Procion-Blue Sepharose 4B (5 cm i.d. × 25 cm) [7] equilibrated with 0.2% Emulgen 911 in A buffer. FMO was eluted with a linear gradient of KCl from 0 to 1.0 M in the equilibration buffer. Fractions containing FMO (0.2–0.3 M KCl) were pooled and dialyzed against equilibration buffer. These were then applied onto DEAE-Sephacel column (Pharmacia Fine Chemical Co., 2 cm i.d. × 10 cm) equilibrated with 0.2% Emulgen 911 in Buffer A, and eluted with a linear gradient of KCl from 0 to 1.0 M in the equilibration

buffer. Fractions containing FMO (0.3–0.4 M KCl) were collected and dialyzed against equilibration buffer. All steps were carried out below 4°.

Determination of catalytic activity of purified FMO. Assays were conducted in the incubation mixture consisting of 1 μmol of substrate (MP or *N*-hydroxy-MP), 15 μg protein of purified FMO, 15 μg of dilauroyl phosphatidylcholine, 0.2 μmol of NADP, 5.0 μmol of glucose-6-phosphate, 0.1 units of glucose-6-phosphate dehydrogenase, 3.0 μmol of $MgCl_2$, 3.0 μmol of *n*-octylamine and 100 mM potassium phosphate (pH 7.4) to make a final volume of 1.0 ml. After incubation for 5 min at 37°, formaldehyde released and *N*-hydroxy-MP formed from MP were determined according to the methods of Nash [8] and Bélanger *et al.* [9], respectively, and *N*-hydroxy-AP formed from *N*-hydroxy-MP was determined as trimethylsilyl derivative by FID-gas chromatography. The conditions used were as follows; column, 5% SE-52 on Chromosorb W (AW, DMCS), 3 mm i.d. × 2 m; column temp., raised from 130 to 180° at a rate of 5°/min; injection port temp., 200°; carrier gas, N_2, 50 ml/min. Retention times of *N*-hydroxy MP and *N*-hydroxy AP in these conditions were 5.3 and 4.8 min, respectively.

Other assays. MP *N*-demethylase and *N*-hydroxylase activities with liver microsomes were determined similarly as described earlier [4]. *N*-Hydroxy-MP *N*-demethylase activity with liver microsomes was measured by determining *N*-hydroxy-AP gas chromatographically as described above. SDS-Polyacrylamide gel electrophoresis was conducted on 9% polyacrylamide slab gels in the presence of 0.1% SDS by the method of Laemmli [10].

Results and discussion

Summary of the purification of guinea-pig liver FMO is shown in Table 1. This preparation exhibited a single band on SDS–polyacrylamide gel electrophoresis and showed a molecular weight (M_r) of about 65,000. FMO has already been purified from liver microsomes of hogs [7, 11], rats [12] and mice [7]. Present preparation from guinea pigs showed slightly higher M_r, but exhibited comparable thiobenzamide *S*-oxidase activity (Table 1) with those of other preparations which were reported to be 340 [13] (hogs) and 1200 [7] to 1420 [13] (mice) nmol/min/mg, respectively.

As shown in Table 2, *N*-hydroxy-MP *N*-demethylase in microsomes was significantly inhibited by methimazole, a potent inhibitor of FMO, but not by SKF 525-A, a strong inhibitor of cyt. P-450, suggesting that this biotransformation was mainly catalyzed by FMO in guinea pigs. For comparison, Table 2 also shows the activities of microsomal MP *N*-hydroxylase and MP *N*-demethylase in the presence of inhibitors. Coutts and Kovach have previously suggested that *N*-hydroxy-MP was nonenzymatically transformed to *N*-[(1-methyl-2-phenyl)ethyl]methanimine *N*-oxide (methanimine *N*-oxide) and *N*-hydroxy-AP, subsequently [14]. In the present study, the purified enzyme showed high activity of *N*-hydroxy-MP *N*-demethylase together with potent MP *N*-hydroxylase and MP *N*-demethylase activities. This means that FMO has high catalytic activity of the formation of methanimine *N*-oxide from *N*-hydroxy-MP. On the other hand, methanimine *N*-oxide, which was synthesized by the method of Coutts *et al.* [5], was readily hydrolyzed and transformed quantitatively to *N*-hydroxy-AP and formaldehyde in 50 mM Tris–HCl buffer at pH 7.4 and at temperatures of either 37° or 4° (data not shown). Thus, FMO was indicated to participate in *N*-hydroxylation of MP and following dehydrogenation. We further found that the resulted *N*-hydroxy AP is further reduced to AP by hydroxylamine reductase containing cytochrome b_5, which will be reported elsewhere [15]. These pathways proposed are illustrated in Fig. 1.

In summary, we purified guinea-pig liver FMO (M_r 65,000) and elucidated its participation in not only *N*-hydroxylation of MP, but also in *N*-demethylation of *N*-hydroxy-MP.

Table 1. Purification steps of flavin-containing monooxygenase from guinea-pig liver microsomes

	Protein (mg)	Total activity* (μmol/min)	Specific activity* (nmol/min/mg)
Microsomes	2240	25	11.2
Solubilized sup.	2016	23	11.4
Blue-Sepharose 4B	62	19	306
DEAE-Sephacel	2.9	4.1	1414

* These activities were monitored by *S*-oxidation of thiobenzamide.

Table 2. Metabolism of methamphetamine and *N*-hydroxymethamphetamine with guinea-pig liver microsomes and reconstituted flavin-containing monooxygenase

	Methamphetamine *N*-hydroxylase	Formaldehyde release from methamphetamine	*N*-hydroxymethamphetamine *N*-demethylase
Microsomes-NADPH	29.7 ± 12.0[a]**	26.0 ± 3.5[b]**	44.1 ± 3.6[c]
+Methimazole (0.25 mM)	3.8 ± 0.5*	9.9 ± 2.8*	27.3 ± 2.5*
+SKF 525-A (0.075 mM)	29.5 ± 9.7	25.0 ± 4.0	37.7 ± 6.2
Flavin-containing monooxygenase	625.0[d]	425.9[e]	1455[f]

[a] Mean ± SEM of four animals; nmol *N*-hydroxy-MP formed/5 min/mg microsomal protein.
[b] Mean ± SEM of four animals; nmol formaldehyde formed/10 min/mg microsomal protein.
[c] Mean ± SEM of four animals; nmol *N*-hydroxyamphetamine formed/30 min/mg microsomal protein.
[d] Mean of four determinations; nmol *N*-hydroxymethamphetamine formed/5 min/mg protein.
[e] Mean of four determinations; nmol formaldehyde formed/5 min/mg protein.
[f] Mean of four determinations; nmol *N*-hydroxyamphetamine formed/5 min/mg protein.
* Significantly different from the control ($P < 0.05$).
** These values from microsomes were quoted from ref. 4.

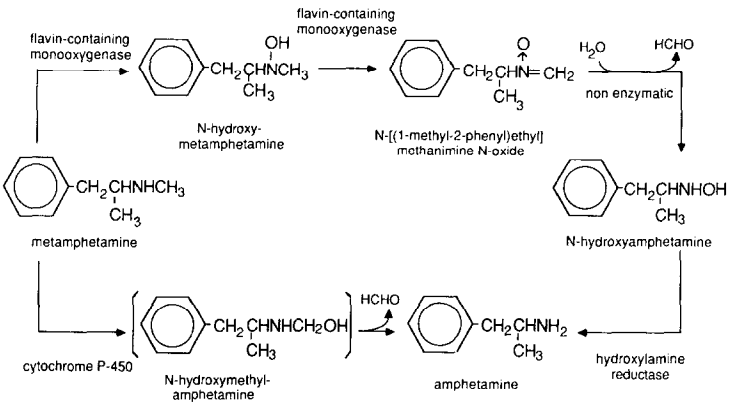

Fig. 1. Proposed metabolic pathway in *N*-demethylation of methamphetamine.

Acknowledgements—We thank Miss R. Ohtsubo, and Messrs K. Makizumi and K. Mohri for their excellent technical assistances.

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Biochemical Pharmacology, Vol. 36, No. 23, pp. 4173–4175, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

Benzodiazepine and $GABA_A$ receptors in rat brain following chronic antidepressant drug administration

(*Received* 16 *April* 1987; *accepted* 23 *June* 1987)

Chronic administration of antidepressant drugs to animals is associated with changes in the number and function of several classes of central monoamine receptors [1, 2]. For example, decreases in the number of cortical β-adrenoceptors and in the sensitivity of the associated noradrenaline-stimulated adenylate cyclase occur after chronic administration of all classes of antidepressant drugs and repeated electroconvulsive shocks [1–5]. Decreases in $5HT_2$ and α_2 adrenoceptor number and function have also been reported although such effects are not seen with all antidepressant treatments [1, 2, 6–8]. Recently cerebral γ-aminobutyric acid (GABA*) receptor subtypes ($GABA_A$ and $GABA_B$) have been studied after chronic antidepressant drugs although the results as yet are inconsistent. Marked increases in $GABA_B$ binding sites in rat frontal cortex have been reported following 18 days administration of several antidepressant drugs by minipump infusion [9, 10]. No reproducible effects on $GABA_A$ receptor binding were found in these studies [9]. However, the numbers of high and low affinity $GABA_A$ binding sites were reduced in the mouse cortex and hippocampus following daily administration of imipramine or nomifensine for 14 days [11]. This effect of chronic administration of antidepressant drugs on $GABA_A$ receptors is supported by a reduction in benzodiazepine (BZ) binding sites [12], a component of the $GABA_A$–ionophore complex. The magnitude of this latter effect is surprising, with decreases of 58–75% in the number of binding sites after desmethylimipramine, zimelidine, buproprion and adinazolam (10 mg/kg twice daily for 21 days). We now report a study of chronic administration of three antidepressants on BZ binding sites in the rat brain. We have also measured concurrently the ability of GABA to stimulate BZ binding.

*Abbreviations used: GABA, γ-aminobutyric acid; BZ, benzodiazepine.