methylation more in the 6–24 hr period than single doses of 5.0, 10.0 or 15 μmol/kg PAD. In the whole 24 hr period rats treated with 2 × 5 μmol/kg PAD had the lowest level of respiratory loss of ^{75}Se, but the difference between the 2 × 5 and 15.0 μmol/kg groups was not significant.

Discussion

Inhibitors of AdoHcy hydrolase through the accumulation of AdoHcy inhibit AdoMet-dependent methylases by a feedback mechanism [9]. Research has been mainly focused on the antiviral and oncostatic properties of these inhibitors [9], but the present study indicates that adenosine dialdehyde, a periodate oxidized adenosine (PAD) exerts well-defined toxic effects in rats. As several low molecular weight compounds, like catechols, norepinephrine, histamine, serotonin and tryptamine, and also macromolecules, like proteins, nucleic acids and membrane phospholipid are substrates for AdoMet-dependent transmethylation [1, 9], the inhibition of transmethylation may affect an arsenal of physiological processes and may produce morphological lesions. Thus haemorrhage in the glandular part of the stomach may have been the consequence of the effect of PAD on the methylation of membrane phospholipid and histamine. One of the characteristics of PAD toxicity is that 5 μmol/kg dose given twice 6 hr apart, caused not only more severe or more widespread hepatic and renal proximal tubular damage than 10 or 15 μmol/kg did, but produced novel effects not seen in single-dosed animals.

Anaemia, haemorrhage in the glandular part of the stomach, enlarged congested liver, bilirubinaemia with signs of jaundice and significantly increased mortality were seen only in rats treated with 2 × 5.0 μmol/kg PAD. Mortality was further increased by selenite. Though the increase was not statistically significant, the low power of the test (see Result section) does not exclude the possibility that selenite increased the lethal toxicity of PAD. This view is supported by the findings of Hoffman and McConnel [4] who have found that mortality occurred only in mice treated with both 4 mg/kg selenite and 100 μmol/kg PAD but not in mice given only one of the two compounds [4].

Considering that the blood volume in rats of 200 g body weight is about 13 ml [10], rats treated with selenite and 2 × 5 μmol/kg PAD had 50.8 × 13 × 0.659 = 0.43 μmol plasma bilirubin. As the decrease in haemoglobin concentration from 13.6 g% to 11.5 g% corresponds to the loss of 17 μmol haemoglobin subunits and the transport maximum for bilirubin excretion is 14 μmol/hr in rats of the same body weight [11], haemolysis was unlikely to be the sole cause of PAD-induced bilirubinaemia. The presence of hydropic degeneration, congestion, periportal damage, cavernous sinusoids and dilated bile duct seen in livers suggests that a defect in the hepatic handling of bilirubin was also a contributory factor.

Comparison of ^{75}Se exhalation between the different dose regimes demonstrates that the group with the lowest degree of dimethylselenide exhalation (and therefore synthesis) had also bilirubinaemia and other signs of increased toxicity. As the inactivation of AdoHcy hydrolase by PAD shows saturability, and the unimolecular reaction between PAD and the enzyme is reversible [9], it seems reasonable to suggest that the total dose is less important in PAD-induced toxicity than the suppression of methylase activity below a threshold level for a prolonged period of time. While 2 × 5 μmol/kg PAD caused a 93% decrease in the exhalation of ^{75}Se, rats given 10 or 15 μmol/kg, PAD had 75 and 79% decrease respectively in the 6–24 hr period.

In summary, 20% of rats given only 10 μmol/kg PAD in two divided doses, but not in a single dose, died within 24 hr and the remaining 80% had loss of haemoglobin, bilirubinaemia, bile duct dilation, severe hepatic congestion and gastric haemorrhage. Selenite, though not statistically significantly, seemed to increase the effects of PAD on lethality, loss of haemoglobin and bilirubinaemia.

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REFERENCES

1. L. R. Bartel and R. T. Borchard, *Molec. Pharmac.* **25**, 418 (1984).
2. E. Marafante and M. Vahter, *Chem-Biol. Interact.* **50**, 49 (1984).
3. S. K. Tandon, L. Magos and M. Webb. *Biochem. Pharmac.* **35**, 276 (1986).
4. J. L. Hoffman and K. P. McConnell, *Archs Biochem. Biophys.* **254**, 534 (1987).
5. J. L. Hoffman, *Archs Biochem. Biophys.* **205**, 132 (1980).
6. S. Siegel, *Nonparametric Statistics for the Behavioural Sciences.* McGraw-Hill Kogakusha, Tokyo (1956).
7. J. C. Cohen, *Statistical Power Analysis for the Behaviour Sciences.* McGraw-Hill Kogakusha, Tokyo (1956).
8. R. B. McCall. *Fundamental Statistics for Psychology.* 2nd Edn, Harcourt Brace Jovanovich, New York (1975).
9. P. M. Ueland, *Pharmac. Rev.* **34**, 223 (1982).
10. E. F. Adolph, *Science, N.Y.* **109**, 579 (1949).
11. I. M. Arias, L. Johnson and S. Wolfson, *Am. J. Physiol.* **200**, 1091 (1961).

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Biochemical Pharmacology, Vol. 36, No. 17, pp. 2867–2869, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Dichloro-*p*-nitroanisole (DPNA)-demethylation not specifically induced by phenobarbitone in mice

(*Received* 26 *September* 1986; *accepted* 5 *February* 1987)

DPNA was introduced as a substrate of the microsomal monooxygenase system of rats by Hultmark *et al.* [1, 2]. Its metabolism was found to be enhanced (calculated per mg microsomal protein) 24-fold after phenobarbitone pretreatment, but only very little after pretreatment with 3-methylcholanthrene. Thus, it seemed to be a substrate of considerable inducer-specificity. When looking for such substrates we investigated DPNA-demethylation after pre-

treatment of different species with inducers of all "types". DPNA proved to be a phenobarbitone (PB)-specific substrate only in rats.

Methods

Animals and treatment. Male C57BL/6J/Han (17–21 g) and NMRI:Han (24–30 g) mice, male Wistar rats (140–190 g) and male guinea-pigs (250–800 g) were used. Maintenance conditions were as described previously [3], guinea-pigs received Höveler Kraftfutter from Höveler Kraftfutterwerke (Langenfeld-Immigrath, FRG). Animals were treated i.p. with the inducers PB, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), prenenolone-16α-carbonitrile (PCN) and β-naphthoflavone (BNF). Doses were: 3 mg TCPOBOP/kg body wt in arachis oil as a single dose 3 days before sacrifice; 80 mg PB/kg body wt and day, guinea-pigs 50 mg/kg, for three days; 30 mg PCN/kg body wt, guinea-pigs 20 mg/kg, suspended in 1% methylcellulose twice a day for 3 days; 80 mg BNF/kg body wt, guinea-pigs 50 mg/kg, in arachis oil for two days.

Synthesis and determination of DPNA. As methylation of 2,5-dichlorophenol and consequent nitration [1] leads to byproducts the separation of which decreases yield considerably, DPNA was synthesized in an alternative way by transforming 2,6-dichloro-4-nitrophenol, solved in ethyl acetate, at slight excess of diazomethane and with fluoroboric acid as catalyst. Ten grams (=0.048 mol) 2,6-dichloro-4-nitrophenol were dissolved in 100 ml ethyl acetate and stood with an excess of diazomethane (0.09 mol), solved in 125 ml ether, at 2–3°. Simultaneously, in order to increase yield, 3–4 drops of fluoroboric acid, as catalyst, were added. After methylation the reaction mixture was filtered, the clear, pale yellow filtrate washed twice with 70 ml 5% K_2CO_3 solution, dried over anhydrous Na_2SO_4 and the solvent mixture (ethyl acetate and ether) evaporated by rotavapor. Two times recrystallizing the yellowish crude product from methanol p.a. affords highly pure 2,6-dichloro-4-nitroanisole as colourless, transparent needles. This procedure yielded 7.8 g (=0.035 mol) (=72.7% of the theoretical yield) highly pure product. It was homogeneous in thin chromatography (DC-SI-F, Riedel de Haen, *n*-hexane/chloroform, 1:1 v/v). Analytical data: melting point, 98–99°; CHN analysis: C, 37.91% (theoretically 37.87%), H, 2.30% (theoretically 2.27%), N, 6.37% (theoretically 6.31%), ^{1}H-NMR (400 MHz) (acetone d_6): $\delta = -8{,}31$ (s, 2 H aromatic), $\delta = 4.03$ (s, 3 H aliphatic); mass spectrum, EI, $m/e = 221$ (M^+ for Cl^{35}).

DPNA demethylation was measured as follows: 1 μmol DPNA was solved in methanol and the solvent allowed to evaporate at 55°. The residue was resolved in 1% bovine serum albumin in 0.07 M phosphate buffer (pH 7.0) and incubated at 37° for 30 min. NADPH regenerating system (6 μmol NAD + 15 μmol $MgCl_2$ + 0.3 μmol NADP + 15 μmol glucose-6-phosphate + 1 Kornberg unit glucose-6-phosphate dehydrogenase in 0.4 ml 0.07 M phosphate buffer, pH 7.0) was added and demethylation started by adding 200 μl microsomal suspension. The reaction was stopped by transferring tubes into an ice bath and precipitating protein by TCA after 10 min. The phenol (and the substrate) were extracted in 2.5 ml CCl_3. Two ml of the organic phase were re-extracted with 2 ml phosphate buffer, pH 7.4. The phenol was measured colorimetrically at 400 nm against a blank which had been passing the whole procedure (but was not added with the ingredients of the regenerating system and microsomes) by means of a calibration curve. Each estimation was performed 3 times in parallel.

All other methodological details were as described previously [3].

Results and discussion

Our findings are listed in two ways: calculated per mg microsomal protein (Table 1a) and per nmol cytochrome P-450 (Table 1b). In rats, there are comparable results to

Table 1(a). DPNA-demethylation in different species after different inducers (2,6-dichloro-*p*-nitrophenol formation in μg per mg microsomal protein in 10 min)

	Mice			Rats	Guinea-pigs
	C57BL/6J		NMRI		
	1st experiment N = 4	2nd experiment N = 4	N = 4	N = 2‡	N = 2‡
Control	4.58	6.11	1.78	2.0	1.09
PB	9.81†	10.99†	5.00†	17.76	4.27
TCPOBOP	9.39†	11.44†	5.31	2.27	1.67
PCN	4.99	5.77	2.90†	0.83	1.49
BNF	—	6.08	3.91†	1.50	1.02

Table 1(b). DPNA-demethylation in different species after different inducers (2,6-dichloro-*p*-nitrophenol formation in μg per nmol cytochrome P-450 in 10 min)

	Mice			Rats	Guinea-pigs
	C57BL/6J		NMRI		
	1st experiment N = 4	2nd experiment N = 4	N = 4	N = 2‡	N = 2‡
Control	6.95	6.42	3.37	4.43	2.11
PB	7.07	4.92	3.09	14.63	3.88‖
TCPOBOP	3.20†	3.85†	2.63§	4.53	4.51
PCN	2.47†	2.57†	1.66*	0.90‖	2.11‖
BNF	—	2.96†	4.69	2.28	1.53

*Significant difference from control ($\alpha \leqq 5\%$).
† Significant difference from control ($\alpha \leqq 1\%$).
‡ Different dates of experiment.
§ Three animals only.
‖ One animal only.

those of Hultmark *et al.* [2]: PB increases DPNA metabolism 9 times, when calculated per mg protein, and 3 times, when calculated per nmol P-450. But in mice, protein-related increase after PB is only 2 times (C57BL/6J) and 3 times (NMRI), and there is no P-450-related increase at all. That means, although PB did augment the total amount of cytochrome P-450, no isoenzyme(s) specific for DPNA-demethylation have been formed. Or: as there is no increase of DPNA-specific activity, DPNA is not a specific substrate for PB in mice.

TCPOBOP even *decreases* DPNA specific activity of cytochrome P-450 (Table 1b) in guinea-pigs. PCN decreases it in mice and rats. BNF decreases it in C57BL/6J/6J- but not in NMRI-mice, and seems to decrease it in rats and guinea-pigs also.

From these results may be drawn the following conclusions. (1) In mice, DPNA is not the prominent marker of PB induction as it is in rats. (2) Although TCPOBOP has been described as PB-like inducer [4] its effect on DPNA metabolism is clearly different from that of PB in C57BL/6J mice and rats. (3) DPNA may be called an atypical substrate, because, in C57BL/6J mice, DPNA-specific activity per cytochrome P-450 is decreased by as different inducers as TCPOBOP ("PB-like"), PCN and BNF ("3-methylcholanthrene-like").

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REFERENCES

1. D. Hultmark, K. Sundh, C. A. Wachtmeister and E. Arrhenius, *Biochem. Pharmac.* **27**, 1129 (1978).
2. D. Hultmark, K. Sundh, L. Johansson and E. Arrhenius, *Biochem. Pharmac.* **28**, 1587 (1979).
3. F. Heubel, T. Reuter and E. Gerstner, *Biochem. Pharmac.* (in press).
4. A. Poland, O. Mak, E. Glover, R. J. Boatman, F. H. Ebetino and A. S. Kende, *Molec. Pharmac.* **18**, 571 (1980).

Biochemical Pharmacology, Vol. 36, No. 17, pp. 2869–2871, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Effect of two inducers of cellular differentiation on the glutathione status of human HL-60 promyelocytic leukaemia and A549 lung carcinoma cells

(*Received* 25 *March* 1987; *accepted* 31 *March* 1987)

Human HL-60 promyelocytic leukaemia cells, undergo differentiation to mature granulocytes when they are incubated with polar solvents such as dimethylsulfoxide (DMSO), dimethylformamide (DMF) or *N*-methylformamide (NMF) at concentrations in the 10^{-1} M range [1]. Exposure to nM concentrations of the tumour promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes maturation to a macrophagic monocytic phenotype [2]. In other cell lines TPA causes growth inhibition apparently without inducing differentiation [3]. This has been observed in, for example, A549 lung carcinoma [4] and A431 epidermoid carcinoma cells [5].

It has been suggested that the induction of differentiation of poorly differentiated tumours by compounds which lack cytotoxic properties may be worthy of investigation as a therapeutic strategy [6, 7]. The biochemical events which trigger chemically-induced cell differentiation *in vitro* are not known. It has been shown recently that DMF and NMF deplete glutathione stores in DLD-1 clone A human colon carcinoma cells without causing toxicity [8, 9]. This effect was observed at concentrations of the solvents which induced the expression of a more benign phenotype. The authors suggested that glutathione may play a role in regulating the growth of these cells. This suggestion has been investigated further in the work described here. In particular, the hypothesis has been tested that glutathione depletion is involved in the mechanism by which agents such as NMF or TPA cause HL-60 cells to differentiate. Glutathione levels have been measured in HL-60 cells before and after chemically-induced maturation. For comparison the effect of NMF and TPA on the glutathione status of A549 cells has been investigated and interpreted in the light of the ability of these compounds to interfere with the growth of A549 cells.

Materials and methods

Materials. TPA was purchased from Sigma Chemical Co. (U.K.), NMF and DMSO from Aldrich Chemical Co. (U.K.); HL-60 cells were obtained from Dr G. Brown, Birmingham University (U.K.) and A549 cells from the American Type Culture Collection (U.S.A.). Cell culture media were purchased from Gibco (U.K.).

Cell culture. HL-60 cells were grown in RPMI 1640 medium with 10% foetal calf serum, A549 cells in Nutrient Hams F12 medium with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (10 pg/ml). HL-60 cells were routinely maintained in logarithmic phase growth between 2×10^4 and $1 \times 10^6 \times ml^{-1}$ by biweekly subculture. A549 cells were subcultured every 5–7 days and the medium was renewed every 2–3 days. All cells were maintained in an incubator at 37° with 5% CO_2. Whereas HL-60 cells grow in suspension, A549 cells adhere to the surface of the culture flask and had to be detached with trypsin (0.1%). Incubations were initiated with either $0.5–1 \times 10^5$ HL-60 cell $\times$ ml^{-1} or 0.5×10^5 A549 cells/flask and included either NMF 180 mM, DMSO 180 mM or TPA 5×10^{-9} M. These concentrations were found to induce maximum differentiation in HL-60 cells. Cells were counted with a haemocytometer or a Coulter Counter; cell viability was assessed by their ability to exclude trypan blue.

Assays for differentiation and glutathione. Functional differentiation of HL-60 cells to macrophagic cell was measured by staining cells for the presence of non-specific esterases [10]; differentiation to granulocytes was assessed by nitro blue tetrazolium reduction [11]. Total glutathione levels (GSH + GSSG) and levels of oxidised glutathione (GSSG) were determined according to Griffith [12].

Results and discussion

Incubation with TPA at nM concentrations causes differentiation in HL-60 cells [2] and growth inhibition in A549 cells [4]. Figure 1 shows that exposure of these cells to 5 nM TPA for 96 hr did not lead to a change in intracellar glutathione levels. In these experiments TPA induced 80–