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Induction of UDP-glucuronosyltransferase isozymes in male and female rat liver microsomes by an isoquinoleine derivative (52028 RP)*

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52028 RP, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide, previously named PK 11195, is an antagonist of the "peripheral type" benzodiazepine binding sites [1], which presents several pharmacological properties on the cardiovascular [2], central [3] and immune systems [4]. 52028 RP was considered as a new model compound to investigate the physiological relevance of "peripheral type" benzodiazepine binding sites [5]. It has been tentatively used as a potential tranquilizer and anticonvulsant in man. Clinical trials and pharmacokinetic studies showed that 52028 RP was metabolized in rat and man very rapidly, and that the rate of metabolism increased upon repeated administration of the drug (A. Uzan, personal communication). This observation led us to the hypothesis that 52028 RP could induce the enzymes involved in drug biotransformation, especially the cytochromes P-450 and the corresponding monooxygenases and UDP-glucuronosyltransferases (UDPGT, EC 2.4.1.17). Indeed, we recently reported in rat that 52028 RP was a potent inducer of different cytochrome P-450 isoenzymes, P-450b (IIB1), P-450p (IIIA1) and P-450j (IIE1), with a different response according to the sex [6].

Concomitant induction of cytochrome P-450 and UDPGT is known to occur upon treatment with various drugs. For example, we found induction of cytochrome P-452 (IVA1) was significantly correlated with that of bilirubin UDPGT, after administration to rats of clofibrate and structurally related compounds [7].

Glucuronidation reactions are selectively enhanced by various inducers such as 3-methylcholanthrene, phenobarbital and hypolipidemic drugs related to clofibrate [8, 9]. Differential induction can be used to relate enzyme activities to several UDPGT isozymes [10]. Indeed after purification by electrofocussing, up to eight or nine isoforms of UDPGT have been isolated in rat liver microsomes [11].

In order to detect which UDPGT isozymes are affected by 52028 RP, the possible inducing effect of the drug was measured in male and female rats. For this purpose the activity of UDPGT towards mono-hydroxylated substrates such as planar and non-planar phenols, monoterpenoid alcohols, steroid hormones, and bilirubin was determined in liver microsomes. The variations in the biosynthesis of the corresponding enzyme proteins were revealed by immunoblots using polyclonal specific antibodies raised against purified UDPGT isoforms from rat liver and kidney.

Materials and methods

Animals. Male and female Sprague–Dawley rats (180–200 g) were obtained from Iffa-Credo (St Germain/l'Abresle, France); they had free access to food (U.A.R., Villemoisson, France) and tap water.

Treatment. 52028 RP (Rhône-Poulenc Santé, Centre de Recherches de Gennevilliers, France) was suspended in a sucrose solution (700 g/l) and then given at a daily dose of 500 mg/kg body wt for 5 days, by gavage to groups of five male and five female rats. For the immunoblot experiments, hepatic microsomes from male and female rats treated with phenobarbital were also used [6]. Control group received the vehicle, only. Microsomal fractions were prepared as previously described [12] and stored as aliquots at −80° until use.

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Biochemical assays. Protein contents were determined with bovine serum albumin as reference, according to the method of Lowry *et al.* [13]. Activity of UDPGT was measured on a fast analyser centrifuge (Cobas, Roche Bioelectronique, Basel, Switzerland) [14]. The aglycones (0.3 mM), dissolved in ethanol/water (1:1 v/v) were 4-nitrophenol, 1-naphthol (Merck, Darmstadt, F.R.G.), umbelliferone, 4-methylumbelliferone, nopol, *cis*-myrtanol, chloramphenicol (Fluka, Buchs, Switzerland), menthol, testosterone, estrone, androsterone (Sigma Chemical Co., St Louis, MO), and morphine hydrochloride salt (Cooperation Pharmaceutique Française, Melun, France). Maximal enzyme activation was obtained with Triton X-100 (Sigma) at a detergent-protein weight ratio of 0.4. Activity toward bilirubin (Merck) was measured according to Heirwegh *et al.* [15] after maximal activation by digitonin (Sigma), at an optimal detergent-protein weight ratio of 1.0.

Immunochemical characterization of UDPGT. Microsomes were analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis [16], using 10% (w/v) acrylamide in the separating gel and 4% (w/v) in the stacking gel. Proteins were transferred electrophoretically with a Trans-Blot system (Bio-Rad, Richmond, CA) at room temperature, for 16 hr at 50 V, in 20 mM Tris–HCl buffer (pH 8.3) containing 20% (v/v) methanol and 150 mM glycine [17]. After protein transfer, nitrocellulose sheets containing protein samples were incubated for the immunochemical detection with goat antisera raised against rat liver or kidney UDPGT [18]. Revelation of the specific bands was obtained upon incubation with the anti-IgG conjugated with alkaline phosphatase (Sigma), using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma) as substrates. Goat antibodies raised against purified renal isoforms recognized UDPGT involved in conjugation of bilirubin and phenols. Antibodies anti-UDPGT from liver presented a broader specificity and recognized at least three hepatic immunoprecipitated proteins active toward bilirubin, phenols and testosterone.

Statistical analysis. The data of enzyme activity were reported as mean ± SD (five animals) and were compared by Student's *t*-test for small samples and non-paired series. Differences between control and treated groups or between male and female rats of $P < 0.01$ were considered significant.

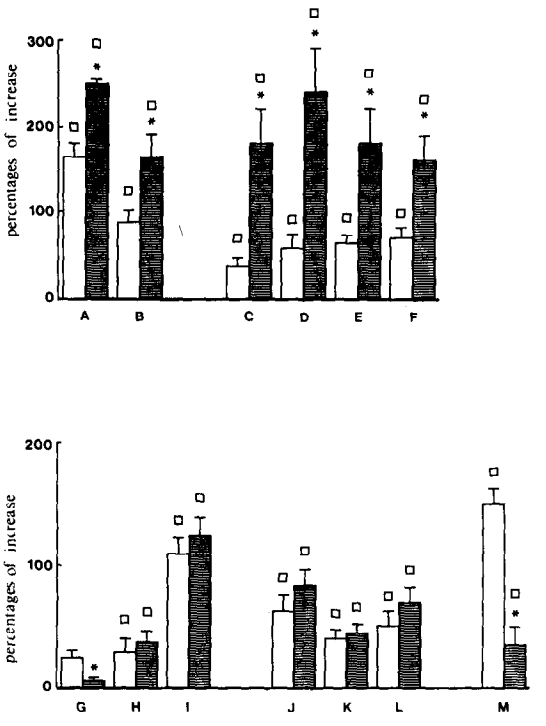

Fig. 1. Effect of treatment by 52028 RP on microsomal UDP-glucuronosyltransferase activities. The activities are expressed in percentage over control values. Control values (nmol/min/mg protein, in male and female rats, respectively) are A, 3.53 ± 0.50, 2.37 ± 0.90 for morphine: B, 3.18 ± 0.20, 2.04 ± 0.90 for chloramphenicol; C, 76.90 ± 2.70, 22.87 ± 1.40 for 1-naphthol; D, 56.85 ± 2.90, 19.53 ± 1.90 for 4-nitrophenol; E, 88.61 ± 5.30, 32.32 ± 4.30 for umbelliferone; F, 91.27 ± 4.10, 39.42 ± 4.40 for 4-methylumbelliferone; G, 12.74 ± 2.10, 6.75 ± 1.00 for androsterone; H, 12.74 ± 0.70, 3.74 ± 0.20 for testosterone; I, 4.63 ± 0.18, 3.34 ± 0.52 for estrone; J, 33.94 ± 2.20, 14.74 ± 0.90 for nopol; K, 15.46 ± 1.00, 7.38 ± 0.40 for menthol; L, 42.47 ± 2.80, 19.17 ± 1.30 for cis-myrtanol; M, 0.79 ± 0.06, 0.86 ± 0.10 for bilirubin. Open and hatched bars correspond to data obtained with male and female rats, respectively. □, significantly different from corresponding controls; *, significantly different from values obtained in male rats.

Results and discussion

The effect of 52028 RP on glucuronidation of various substrates is reported in Fig. 1. The drug significantly enhanced, whatever the sex, the glucuronidation of all substrates used, except androsterone. Glucuronidation of the planar mono-hydroxylated phenols 1-naphthol, 4-nitrophenol, umbelliferone and 4-methylumbelliferone was respectively increased by 37, 57, 63 and 69% in treated males when compared to the corresponding controls (Fig. 1). These differences were even larger in females. Similar pattern was observed when glucuronidation of bulkier structures such as chloramphenicol and morphine was considered, reaching 249% increase for morphine in female rats. Bilirubin glucuronidation was also stimulated by 52028 RP, with 148 and 33% increases in male and female rats, respectively. Conjugation of the monoterpenoid alcohols *cis*-myrthanol, menthol, nopol and the steroid hormones testosterone and estrone was also significantly increased after treatment, but no significant difference could be observed according to the sex (Fig. 1).

These results emphasize multiple effects of the drug on UDPGT isozymes. The increase in activity was probably due to an induction process. Several following points could account for such conclusion. Protein activation is not likely to occur, since an *in vitro* addition of 52028 RP to the microsomes did not change the enzyme latency (results not shown). We recently reported that 52028 RP was responsible for a strong hepatomegaly (45%, 67% increases in liver weight in male and female rats, respectively), associated to increase (58 to 68%) of total microsomal proteins in the two sexes, when compared to the controls [6].

Finally the results of the immunoblot experiments (Fig. 2) favoured an induction phenomenon. Figure 2a shows the immunostaining obtained with antibodies raised against the renal purified UDPGT isoforms involved in conjugation of bilirubin and phenols. This antibody recognized two main bands in control, phenobarbital- and 52028 RP-treated rats, whatever the sex. The lower major band of 54 kD corresponds to the enzyme proteins active toward bilirubin and phenols. The staining of this band was strongly

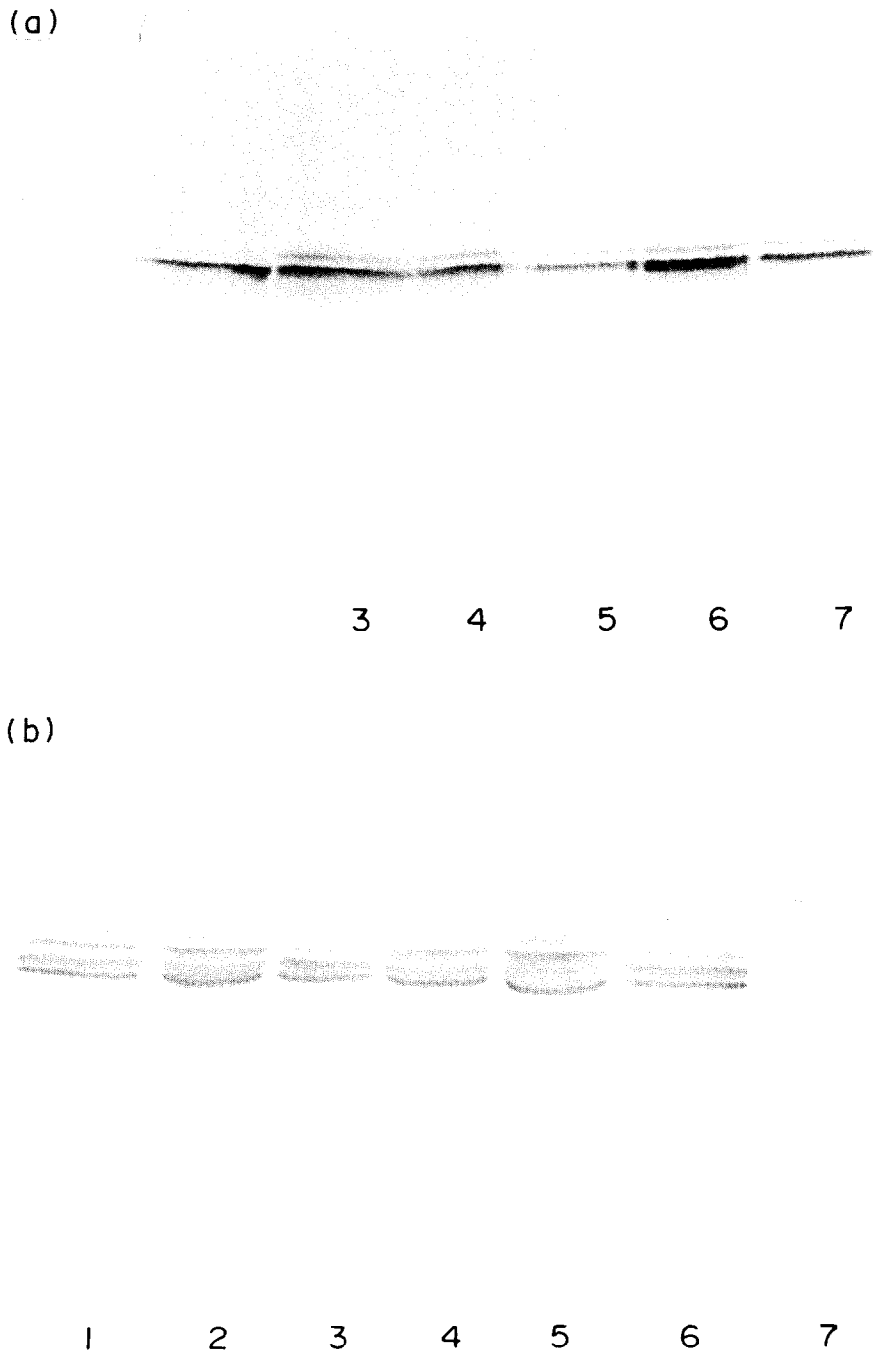

Fig. 2. Immunoblotting revelation of UDP-glucuronosyltransferases after induction by 52028 RP using IgG raised against purified rat kidney (a) or liver (b) enzyme. For each sample, 20 μg proteins were loaded onto the gel. (a) Lanes 1, molecular weight standards; lanes 2 to 4, microsomes from control, 52028 RP- and phenobarbital-treated male rats, respectively; lanes 5 to 7, microsomes from control, 52028 RP- and phenobarbiral-treated female rats, respectively. (b) Lanes 1 to 3, microsomes from phenobarbital-, 52028 RP-treated and control female rats; lanes 4 to 6, microsomes from phenobarbital-, 52028 RP-treated and control male rats; lane 7, molecular weight standards. A schematic representation for the recognition of isozymes is given in Ref. 18.

enhanced in 52028 RP- and phenobarbital-treated rats. On the other hand, the antibody also recognized another immunoreactive protein (56 kD), whose catalytic activity has not been assigned yet, present in all male and female microsomal samples investigated. Coughtrie *et al.* [18] have previously demonstrated the existence of this unknown band in male adult rats. The staining of this minor band was also increased after administration of 52028 RP. A Western blot was also carried out using an antibody with broad specificity raised against rat liver UDPGT (Fig. 2b).

This antibody recognized four immunoreactive proteins: an enzyme active toward testosterone (50 kD), toward bilirubin and phenols (54 kD), and two additional proteins whose specificity is not known yet (56 and 52 kD) [18]. The immunostaining revealed mainly tht 54- and 56 kD-bands, whose intensity was clearly enhanced upon treatment with the drug in both sexes (Fig. 2b). From its wide inducing effect observed on the various UDPGT isoenzymes, 52028 RP resembles phenobarbital in that respect. The antiepileptic drug, besides a strong hepatomegaly, is known to stimulate preferentially glucuronidation of terpenes, morphine, chloramphenicol, bilirubin and testosterone [8, 19], whereas that of planar phenols is only slightly enhanced or not affected. This assumption was corroborated by the fact that 52028 RP also enhanced the concentration in cytochrome P-450b (IIB1), which corresponds to the main form induced with phenobarbital [20]. Moreover, the cytochromes P-450p (IIIA1) and P-450j (IIE1), which are selectively induced by pregnenolone 16α-carbonitrile and ethanol or isoniazid, respectively [21, 22] were also increased by the drug. This suggests that 52028 RP had a non-specific inducing effect more pronounced than phenobarbital. Interestingly, the drug did not affect the content in cytochrome P-450c (IA1) but increased glucuronidation of planar phenols substantially. Glucuronidation of these substrates is markedly stimulated by 3-methylcholanthrene. The two inducing processes for cytochrome P-450c and UDPGT isozyme active toward planar phenols are known to be mediated through interactions of 3-methylcholanthrene-type inducers with the *Ah* locus [23]. Thus, if 52028 RP presented phenobarbital-like inducing effects, the two drugs differed from each other namely by their actions on the stimulation of planar phenol glucuronidation.

In summary, the increases in glucuronidation of various substrates after treatment and the analysis of immunoreactive proteins indicate that the new isoquinoleine derivative 52028 RP is a potent inducer of various UDPGT isozymes in liver of male and female rats. Differences in the induction level was observed according to the sex, for several substrates considered. Compared to phenobarbital, 52028 RP is a more powerful inducer of UDPGT.

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Centre du Médicament
U.R.A. CNRS No. 597,
Faculté des Sciences
Pharmaceutiques et
Biologiques
30 rue Linnois
54000 Nancy, France

MURIEL TOTIS
JACQUES MAGDALOU
SYLVIE FOURNEL-GIGLEUX
ANNE-MARIE BATT*
GÉRARD SIEST†

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* To whom correspondence should be addressed.
† With the technical collaboration of Christine Masson.

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Uptake of antimalarial bis(benzyl)polyamine analogs by human erythrocytes

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It is known that the natural polyamines putrescine, spermidine and spermine [1, 2], as well as a number of polyamine analogs [2–5], are accumulated in various cells, including human erythrocytes [6], by a carrier-mediated mechanism. Polyamine transport into most cells has also been shown to be energy dependent [7]; however, energy dependence for polyamine uptake into human erythrocytes has not been demonstrated unambiguously [1, 6]. We recently reported on the growth inhibitory effects of a series of bis(benzyl)polyamine analogs on the erythrocytic stages of both human (*Plasmodium falciparum*) and rodent (*Plasmodium berghei*) malaria parasites [8]. Because our new antimalarial agents are polyamine analogs, it was of interest to explore the possibility that these novel antimalarial polyamines were accumulated in erythrocytes by the uptake mechanism used for the natural polyamines.

In the present report we show that antimalarial bis(benzyl)polyamine analogs (e.g. *N,N'*-bis[3-[(phenylmethyl)amino]propyl]-1,8-diaminooctane; MDL 27391) are concentrated by human erythrocytes *in vitro*. The accumulation process shows saturation kinetics and is apparently carrier-mediated. However, the uptake system may be distinct from the transport system responsible for uptake of the natural polyamines and other polyamine analogs described previously. This drug uptake system may, in part, determine the efficacy of the novel polyamines against malaria parasites.

Methods

Preparation of erythrocytes. Venous blood from two of the authors (A.J.B. (B+) and J.A.D. (A+) was used in all experiments. The blood was anticoagulated with a citrate:phosphate:dextrose solution and washed three times by centrifugation and resuspension using RPMI 1640 medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.2). Washed erythrocytes were stored as a 50% suspension in the wash buffer at 4° for no more than 1 week, during which time ATP levels in the erythrocytes, as measured by a bioluminescence assay [9], remained constant.

Polyamine and polyamine analog uptake by erythrocytes. Uptake experiments with erythrocytes were carried out as described by Moulinoux *et al.* [6]. Erythrocytes were washed three times in 0.14 M NaCl and resuspended in the same solution prior to uptake measurements. Erythrocyte suspensions (2×10^9 in 0.3 ml) were mixed with 0.3 ml of homologous human serum, [^{14}C]-spermidine or a [^{14}C]-polyamine analog and various competitive inhibitors or other drugs to make a final volume of 0.63 ml for uptake incubations. The pH of the incubations under these conditions was 7.4. Identical results for uptake of polyamines into erythrocytes were obtained if RPMI 1640 medium supplemented with 10% human serum and 25 mM HEPES (pH 7.4) was substituted for the 0.14 M NaCl/serum mixture. Some experiments were carried out using RPMI 1640 medium with 10% human serum and 25 mM (piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.5).

Uptake incubations were done at 37° with vigorous shaking in a water bath for either 15 or 30 min at pH 7.4 or pH 6.5, respectively, and then terminated by adding 2.5 ml of ice-cold 0.14 M NaCl and sedimenting the erythrocytes by centrifugation for 4 min in the cold. The cells were washed twice more with 2.5 ml of ice-cold 0.14 M NaCl and then lysed with two packed cell volumes (0.4 ml) of distilled water followed by precipitation of protein in the hemolysate with two packed cell columns (0.4 ml) of ice-cold 10% perchloric acid. Samples were then centrifuged at 15,000 *g*, and 0.5-ml aliquots of the supernatant fractions were taken for determination of radioactivity by liquid scintillation counting in 10 ml of Aquasol. Uptake was linear for 30 min at pH 7.4 and at least 60 min at pH 6.5.

Polyamine analog analysis by HPLC. Polyamine analogs were measured in erythrocytes by derivatization with dansyl chloride and separation by HPLC as described previously [10].

Chemicals. [Tetramethylene-1,4-^{14}C]-Spermidine (80 mCi/mmol) was purchased from New England Nuclear. Other reagents were purchased from Sigma. All polyamine analogs including *N,N'*-bis[3-[(phenylmethyl)amino]-propyl]1,8-octane-^{14}C]-diamine (48 mCi/mmol) were synthesized at the Merrell Dow Research Institute.

Results

A series of antimalarial bis(benzyl)polyamine analogs, differing only in the length of the central methylene chain, were accumulated by human erythrocytes during 24 hr of culture at 37° (Table 1). Concentrations of polyamine analogs within the erythrocytes were four (MDL 27693) to twenty-four (MDL 27701) times higher than the concentrations found in the culture medium after 24 hr. A polyamine analog with free terminal amines (MDL 26547) rather than terminal benzyl groups was neither taken up nor concentrated by the erythrocytes. Thus, the terminal