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DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—IV

INVESTIGATION OF THE DIFFERENCES IN RESPONSE OF ISOLATED LIVER CELLS AND THE LIVER OF THE INTACT CHICK EMBRYO TO PORPHYRIA-INDUCING DRUGS*

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Abstract—3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine induces porphyrin biosynthesis in monolayer cultures of chick embryo liver cells, but not in the intact chick embryo while 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine induces porphyrin biosynthesis in both systems. To investigate the reason for this finding, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine-^{14}C were prepared, injected into 17-day-old chick embryos and the total amount of drug and metabolite(s) in the livers measured at various time periods. 3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine was found to undergo a more rapid metabolic degradation in liver than 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine, and its inactivity in the chick embryo was attributed to its rapid metabolic degradation. The fact that 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine causes porphyrin accumulation in chick embryo liver cells is due to the inability of the cells to rapidly metabolize the drug.

RECENTLY Granick[1] devised a procedure for screening drugs for porphyria-inducing activity. The method consists of adding drugs to a monolayer of chick embryo liver cells grown on coverslips. The active drugs induce increased synthesis of δ-aminolevulinic acid (ALA) synthetase resulting in the accumulation of porphyrins which are readily visualized with the fluorescence microscope. Granick[1] found a large number of compounds to induce porphyrin biosynthesis in cell culture. Among these were glutethimide, the barbiturates, phensuximide, methsuximide, and griseofulvin. On the basis of these results Granick[2] cautioned against the use of these compounds in patients with porphyria and relatives of these patients. Racz and Marks[3] questioned the validity of excluding drugs on the basis of results in a cell culture system and compared the ability of a number of drugs to induce porphyria when injected into the fluids surrounding the chick embryo with results obtained in liver cell culture.

3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) exhibited porphyria-inducing activity in monolayer cultures of chick embryo liver cells and in the intact chick embryo. The corresponding pyridine, 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (Ox-DDC) was practically devoid of activity in the intact chick embryo whereas in monolayer cultures of chick embryo liver cells it exhibits slightly less potency but greater efficacy than DDC. Ox-DDC was also inactive when administered orally to guinea pigs, and this was attributed to dynamic phenomena which control drug concentration in the liver.[4] In view of the structural similarity of the two compounds it was difficult to reconcile the fact that both DDC and Ox-DDC were

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active in monolayer cultures of chick embryo liver cells but only DDC was active *in vivo*.

There were two possible reasons for this result: (1) after administration to animals, Ox-DDC did not reach the liver in sufficient quantities to induce porphyrin production; and (2) Ox-DDC was rapidly metabolized and inactivated in the liver when administered to animals. In this paper we describe experiments designed to examine these two possibilities.

EXPERIMENTAL

Fertilized eggs used were of a White Leghorn strain obtained from a local hatchery and stored at 10° for no longer than 7 days prior to incubation at 38°. The age of the embryo was taken as the number of days from onset of incubation.

Ethyl acetoacetate-3-^{14}C (4·17 mc/m-mole) was purchased from New England Nuclear Corp. Melting points are uncorrected.

Instrumentation

Gas–liquid chromatography was carried out with a Microtek Model 2000MF gas Chromatograph fitted with a 5-ft stainless steel column packed with celite. The hydrogen flame ionization detector and helium carrier gas were used for all separations. A Bausch and Lomb Spectronic 505 was used for recording ultraviolet spectra. All radioactive samples were counted in a Nuclear Chicago Model 6850 Liquid Scintillation System. Counts were corrected for quenching by the sample channels ratio method and the background count subtracted. The thin-layer chromatograms containing radioactive samples were scanned using a Nuclear Chicago Actigraph III radiochromatography system. All radiochromatograms were scanned at a detector voltage of 960 V and gas pressure of 7 lb.

Synthesis of labeled compounds. (a) DDC-^{14}C. DDC-^{14}C was prepared by a general method described for the unlabeled compound by Loev and Snader.[5] Redistilled ethyl acetoacetate (0·002 mole) was added to a vial containing ethyl acetoacetate-3-^{14}C (4·17 mc/m-mole) to give a product of 0·048 mc/m-mole. The contents of the vial were transferred to a 5-ml boiling flask with the aid of 2 ml of ethanol. Acetaldehyde (0·001 mole) and 0·002 mole of ammonia solution were added and the mixture refluxed for 3 hr. After cooling, the solution was poured into 15 ml of ice-cold 0·1 N HCl whereupon the product crystallized (yield 65 per cent). The product was collected and purified by successive crystallizations from ethanol–water until the specific activity of the compound remained constant. This was achieved after three crystallizations. The product, m.p. 128·5–129, was found to have a specific activity of 0·088 mc/m-mole, λ_{max} (ethanol) 232 and 351 mμ (ϵ 18,100 and 8100). The product was found to be homogeneous by gas–liquid chromatography.

(b) Ox-DDC-^{14}C. For the preparation of Ox-DDC-^{14}C, 1 g of DDC-^{14}C (0·004 mole; 0·010 mc/m-mole) was dissolved in 10 ml of glacial acetic acid in a round bottom flask and sodium nitrite (1 g) added in small portions over a period of 1 hr with constant stirring. After stirring for an additional 90 min the mixture was poured into 100 ml of ice-cold water and the product which separated as an oil extracted with three 50-ml portions of ether.[5] The product was extracted from the ethereal solution with three successive portions (20 ml) of 10% HCl. Following neutralization of the acidic solution

with sodium carbonate, the aqueous phase was extracted with three 50-ml portions of ether. The ethereal solution was dried over sodium sulfate and the ether removed. Distillation (bulb-tube) afforded Ox-DDC-^{14}C as a colorless oil, b.p. 105–115/0·07 mm, λ_{max} (ethanol) 269·5 mμ (ϵ 3,040). The yield was 70 per cent. Marks *et al.*[4] reported λ_{max} (ethanol) 269 mμ (ϵ 3,250). The specific activity of this compound was found to be 0·010 mc/m-mole. The product was found to be homogeneous by gas–liquid chromatography.

Amount of ^{14}C in the livers at different time intervals after injection of ^{14}C labeled drugs into the fluids surrounding the chick embryo. An accurately weighed amount of labeled drug was mixed with unlabeled drug and the mixture dissolved in a quantity of dimethylsulfoxide calculated to give a dilution of drug such that the amount of drug to be injected was contained in 0·1 ml of solution. The drug dissolved in 0·1 ml of dimethylsulfoxide was then injected into 17-day-old chick embryos as previously described.[3] At the end of various incubation periods the livers of the chick embryos were removed, washed with 2 ml of saline to remove any drug absorbed to the surface of the liver, weighed and placed on a piece of cellophane (2 × 3 in.) where they were dried under an infrared heat lamp for 6 hr. The dried tissue was prepared for liquid scintillation counting by the oxygen flask combustion technique.[6] The recovery of known amounts of DDC-^{14}C added to livers from control chick embryos was 99·9 $\pm$ 0·9 per cent ($\pm$ S.E. Mean, $n = 10$).

Amount of unchanged drugs in the liver at different time periods after injection of the labeled drugs into the fluids surrounding the embryo. The labeled drugs (DDC-^{14}C and Ox-DDC-^{14}C) were administered as described above. The livers were removed, washed several times with saline and then stored frozen until the extraction of the drug and its metabolites could be carried out. The liver was weighed, homogenized in 3 ml of methanol in a Potter–Elvehjem apparatus and the homogenate transferred to a centrifuge tube. After centrifugation, the supernatant was decanted into a small flask and the residue resuspended in an additional 3 ml of methanol for homogenization and centrifugation. The extraction procedure was repeated and the three supernatants combined. The tissue residue was allowed to dry in air, combusted and counted. No significant amount of radioactivity was found to be present in this residue. The methanol extract containing the radioactive materials was reduced to a volume of 0·5 ml and an aliquot (0·3 ml) spotted as a band on a 2-in. thin-layer plate coated with a 250 micron layer of Silica gel G. The plate was developed with benzene–methanol (14:1).[7] The R_f values for DDC and Ox-DDC were found to be 0·45 and 0·72. The thin-layer plates were scanned for detection of the radioactive bands with a Nuclear Chicago Actigraph III radiochromatogram Scanner and the radioactive portions of Silica gel G removed and counted by the following procedure: the radioactive Silica gel portions were placed in liquid scintillation vials containing 0·45 g of Cab-O-Sil and 15 ml of toluene scintillation solution. The scintillation solution was prepared by dissolving 6 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) in 1 liter of toluene. The vial was agitated to suspend the Silica gel and counted. In the case of the experiments with Ox-DDC the area of Silica gel corresponding to an R_f value of 0·72 was removed and counted even though no radioactivity could be detected with the scanner, since small quantities of radioactivity would escape detection by the scanner but not by liquid scintillation counting.

Determination of δ-aminolevulinic acid synthetase activity. DDC (4 mg) was dissolved

in dimethylsulfoxide (0·1 ml) and injected into 17-day-old chick embryos as previously described.[3] At the completion of the incubation period the embryo was sacrificed and the liver removed, blotted, weighed and homogenized in 3 vol. of 0·9% sodium chloride solution containing 0·5 mM EDTA and 10 mM tris, pH 7·4.[8] Aliquots (0·5 ml) of the homogenate were added to each of two Erlenmeyer flasks (25 ml) containing 1·5 ml of a solution at pH 7·2 of the following constitution: 200 μmoles of glycine, 20 μmoles EDTA and 150 μmoles of tris-HCl.[8] One ml of 25% trichloroacetic acid was added to one of the flasks which was to serve as a blank. The two flasks were incubated at 37° for 1 hr in a shaker bath and 1 ml of 25% trichloroacetic acid added to the test flask. The precipitates were removed by centrifugation and 2 ml of supernatant added to 1 ml of 1 M phosphate buffer, pH 6·8, in test tubes.

After adding 6 drops of 5 N NaOH and 0·1 ml of ethyl acetoacetate to each tube, the tubes were stoppered and heated at 100° for 10 min. Modified Ehrlich reagent[9] was added and the optical density of the test sample determined at 552 mμ relative to the blank. It was shown by means of a solvent partition technique[1] and standard reference pyrroles that the pyrrole estimated by this technique was ALA-pyrrole uncontaminated by aminoacetone-pyrrole.* The absorption spectrum of the complex between the pyrrole and Ehrlich reagent was identical to that obtained with an authentic specimen of ALA-pyrrole.

The same procedure was used to study the δ-ALA synthetase activity after injection of Ox-DDC (4 mg) in dimethylsulfoxide (0·1 ml) into the chick embryo.

Demonstration of presence of active drug in cell culture medium 24 hr after incubation. Chick embryo liver cells were grown as monolayers in eight, 5-cm Petri dishes as described by Granick.[1] After 24 hr of growth the medium was changed and the cells incubated with 50 μg/ml of Ox-DDC for a further 24-hr period. The porphyrin content of the media for four dishes was then determined. The media from the remaining four dishes was transferred to cells grown in drug free medium and after 24 hr of incubation the porphyrin content of cells and media determined.[1] The presence of active drug in the media, which had been transferred, was revealed by demonstrating the accumulation of porphyrins in the cells and media.

TABLE 1. PORPHYRIN ACCUMULATION IN CHICK EMBRYO LIVER CELLS AS A RESULT OF REMAINING DRUG IN MEDIA PREVIOUSLY EXPOSED TO CELLS*

Drug concentration (μg/ml)	Porphyrin accumulation (μg/mg protein)†
Control (solvent only)	0·022 (0·019–0·025)
10	0·026 (0·016–0·026)
20	0·056 (0·049–0·061)
50	0·106 (0·087–0·132)

* The extreme values are given in parentheses.
† Expressed as coproporphyrin I/mg protein.

* D. L. Tyrrell, personal communications.

This experiment was repeated with 0, 10 and 20 μg/ml Ox-DDC. Ox-DDC solutions in ethanol were used containing 10, 20 and 50 mg/ml. In this way the drug was always added to the media in 5 λ of ethanol. The results are shown in Table 1 and are expressed as μg coproporphyrin/mg protein.

RESULTS AND DISCUSSION

The first objective of our studies was to synthesize DDC-^{14}C and Ox-DDC-^{14}C in radiochemically pure form. There was no difficulty in obtaining a radiochemically pure sample of DDC-^{14}C since the compound was a solid and could be crystallized to constant specific activity. In the case of Ox-DDC-^{14}C, synthesized by oxidation of DDC-^{14}C with nitrous acid, it was more difficult to establish radiochemical purity because it was an oil and too small a quantity was available for repeated distillation. To overcome this problem the radiopurity of Ox-DDC-^{14}C was assessed by comparing its specific activity to that of the material from which it was prepared, viz. DDC-^{14}C. The specific activities were found to correspond within the limits of experimental error.

The second objective of our study was to establish a procedure for converting the colored hepatic tissue, containing radioactive substances into a form suitable for counting. The oxygen flask combustion method was found to possess several advantages over the conventional procedures in which organs are solubilized with hyamine or similar materials.

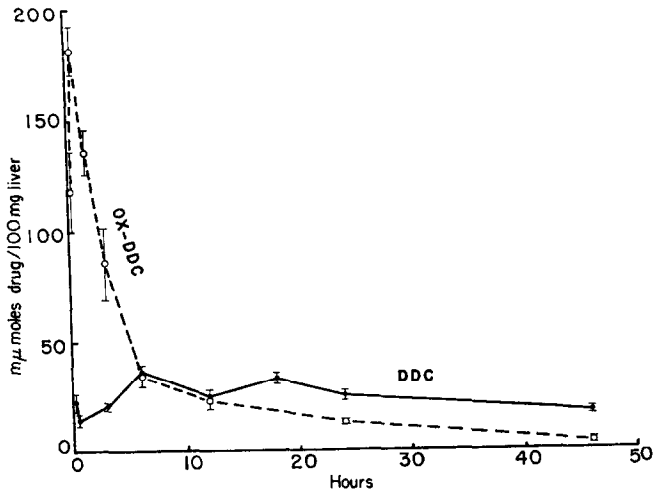

FIG. 1. Amount of radioactive drug in livers of 17-day old chick embryos at different time intervals after injection of DDC-^{14}C (●———●) and Ox-DDC-^{14}C (○- - - -○). Each point represents mean ± S.E. of mean.

The third and primary objective of this study was to measure the total amount of radioactivity and unchanged drugs in the livers of 17-day-old chick embryos at various time intervals after injection of DDC-^{14}C and Ox-DDC-^{14}C into the fluids surrounding the embryo. The total amount of radioactivity in the livers of chick embryos at different time intervals after injection of DDC-^{14}C and Ox-DDC-^{14}C is shown in Fig. 1. The amount of radioactivity in the liver is expressed as mμmoles of drug/100 mg liver. The amount of radioactivity after injection of Ox-DDC-^{14}C reached a maximum in the

liver after approximately 0·5 hr and then dropped rapidly. The amount of radioactivity after injection of DDC-^{14}C reached a maximum in the liver after approximately 6 hr and remained elevated for a considerable period of time. Since the radioactivity from Ox-DDC-^{14}C attained a level in the liver approximately five-fold higher than that reached by the radioactivity from DDC-^{14}C, it is clear that the inactivity of Ox-DDC in the intact chick embryo cannot be ascribed to an inability to reach the liver in sufficient quantity to induce porphyrin formation.

These results indicate, however, that Ox-DDC may be metabolized and excreted so rapidly that the drug was not present in an adequate concentration in an unchanged form for a sufficient period of time to induce porphyria. To examine this possibility, the total radioactive material in the livers of 17-day chick embryos was extracted at various time intervals after injection and separated by means of thin-layer chromatography. In the case of DDC-^{14}C at 6 and 24 hr two radioactive areas were detected, one at the origin and the other with an R_f corresponding to that of DDC (Fig. 2). In the case of Ox-DDC-^{14}C at 6 and 24 hr, only one radioactive area could be detected

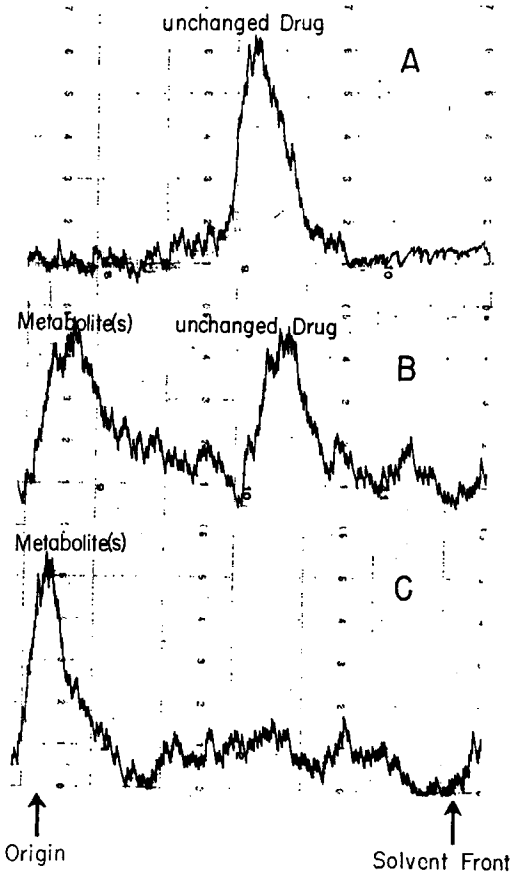

FIG. 2. Metabolism of DDC-^{14}C; Radiochromatogram Scanner records showing DDC-^{14}C added to control liver (A), liver extract 6 hr after injection of DDC-^{14}C (B), and liver extract 24 hr after injection of DDC-^{14}C (C).

with the radiochromatogram scanner which had an R_f different to that of the original compound (Fig. 3). The results (Fig. 4) show that 30 min after the administration of Ox-DDC (4 mg; 15,000 mμmoles) there is 72·5 mμmoles of drug in 100 mg of liver. The drug level drops rapidly to 5·3 mμmoles in 100 mg of liver at 1·5 hr and at 6 hr is barely detectable. On the other hand, 6 hr after the administration of DDC (4 mg; 15,000 mμmoles) there is 9·9 mμmoles of drug in 100 mg liver, and the drug level drops relatively slowly thereafter and is present in detectable amounts for at least 36 hr after injection.

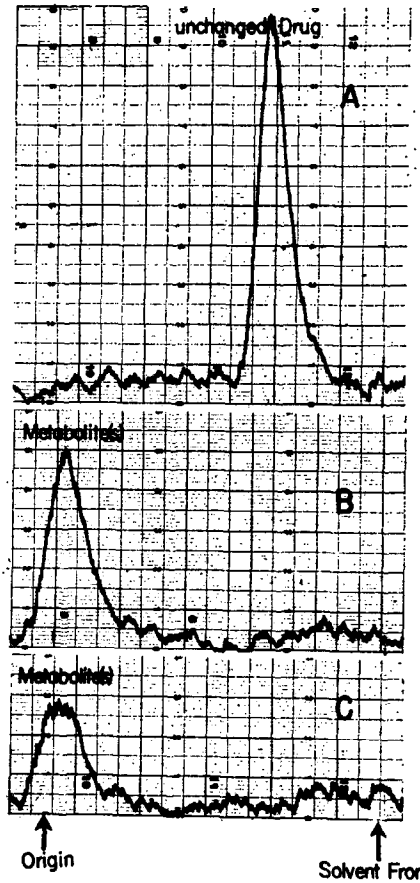

FIG. 3. Metabolism of Ox-DDC-^{14}C; Radiochromatogram records showing drug added to control liver (A), liver extract 6 hr after injection of drug (B), and liver extract 24 hr after injection of drug (C).

These results clearly demonstrate that Ox-DDC undergoes more rapid metabolism and excretion in liver than does DDC. Thus it appeared likely that the inactivity of Ox-DDC in the intact chick embryo was due to its rapid uptake, degradation and excretion. This idea was supported by the following observations: following injection of DDC and Ox-DDC into 17-day-old chick embryos DDC caused a marked increase in δ-aminolevulinic acid (δ-ALA) synthetase activity which reached a maximum in

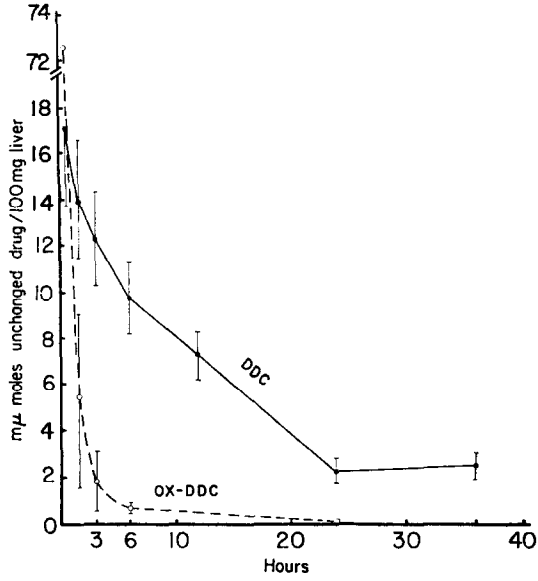

FIG. 4. Amount of unchanged drug in livers of 17-day-old chick embryos at different time intervals after injection of DDC-^{14}C (●———●) and Ox-DDC-^{14}C (○- - - -○). Each point represents mean ± S.E. of mean.

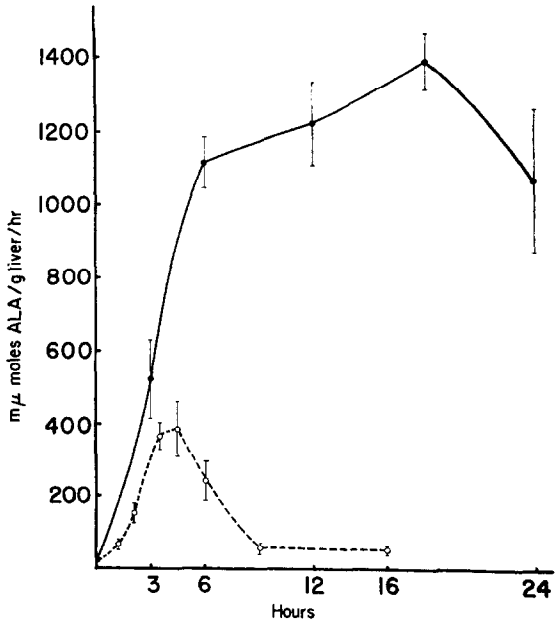

FIG. 5. δ-ALA synthetase activity in livers of 17-day-old chick embryos at different time intervals after injection of DDC (●———●) and Ox-DDC (○- - - -○). Each point represents mean ± S.E. of mean.

6 hr and was still elevated at 24 hr (Fig. 5). With Ox-DDC a considerably lower maximum in δ-ALA synthetase activity was observed in 4 hr and control levels were found in 8 hr (Fig. 5). In the Ox-DDC-treated chick embryo no porphyrin accumulation was observed after 8 hr. The lack of porphyrin accumulation with Ox-DDC is thus attributable to rapid degradation and excretion so that δ-ALA synthetase levels are not sufficiently elevated for an adequate period of time.

It appeared probable that Ox-DDC causes porphyrin accumulation in chick embryo liver cell culture due to the inability of the cells to metabolize and inactivate Ox-DDC at a sufficient rate to prevent it exerting its porphyria-inducing activity. This idea was supported by the demonstration of residual active drug in the liver cell culture medium 24 hr after the beginning of the incubation period (Table 1) in contrast to the absence of Ox-DDC from the chick embryo liver 24 hr after drug administration. A further question of interest raised by these results is the following. It has recently been suggested that liver cell cultures might be used as a system for screening drugs for hepatotoxicity.[10] Since it is probable that drugs are metabolized at a slow rate in cell culture, some false positive results may be obtained. On the other hand screening in liver cell culture reveals a potentiality for damage in the liver, which becomes important when drug-metabolic pathways become limiting in a variety of disease states.

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