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Reduction of the Potential Anticancer Drug Oracin in the Rat Liver In-vitro

BARBORA SZOTÁKOVÁ, LENKA SKÁLOVÁ, VLADIMÍR WSÓL AND EVA KVASNIÈČKOVÁ

Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic

Abstract

Studies on the metabolism of the potential cytostatic drug oracin have shown that a principal metabolite of oracin is 11-dihydrooracin (DHO). We conducted in-vitro experiments to investigate the extent of oracin carbonyl reduction in microsomal or cytosolic fractions and to find out the enzymes involved under these conditions.

Among several inducers of rat cytochrome P450 only 3-methylcholanthrene caused a significant ($P < 0{\cdot}01$) stimulation (1·9 times) of DHO production in microsomal fraction and the specific P4501A inhibitor α-naphthoflavone significantly ($P < 0{\cdot}01$) decreased (twice) the induced reduction activity. Cytochrome P4501A participates in oracin reduction in microsomes. 18β-Glycyrrhetinic acid, a specific inhibitor of hydroxysteroid dehydrogenase, significantly ($P < 0{\cdot}01$) inhibited the production of DHO in the microsomal fraction (>95% inhibition) in comparison with the non-inhibited reaction. Statistically significant ($P < 0{\cdot}01$) inhibition (95%) of DHO formation was caused by metyrapone, which is also the substrate of 11β-hydroxysteroid dehydrogenase.

The main microsomal enzyme which catalyses the carbonyl reduction of oracin is probably 11β-hydroxysteroid dehydrogenase. Important oracin reduction to DHO in the cytosolic fraction was found. According to its specific sensitivity towards quercitrin (inhibition by 99%, $P < 0{\cdot}01$), the enzyme responsible for DHO formation in the rat liver cytosol is postulated to be carbonyl reductase.

Carbonyl-containing xenobiotic compounds are reduced in mammals by pyridine nucleotide-dependent reductases (Felsted & Bachur 1980). Cytosolic fractions predominantly express high specific activity but the membranes also contain considerable amounts of carbonyl reductase activity (Oppermann et al 1991). These enzymes can be distinguished functionally by the use of model substrates and their sensitivity to specific inhibitors (Felsted & Bachur 1980; Büld et al 1996).

Although cytochrome P450 is generally recognised as a monooxygenation catalyst, it is also necessary to take into account its ability to catalyse the reduction of xenobiotics. In the so-called xenobiotic reductase activity of P450, certain xenobiotics may accept electrons from the reduced [Fe^{2+}-P450] complex in the absence of oxygen. Classic P450 inhibitors are also potential inhibitors of xenobiotic reductase activity either by competing with the xenobiotics at the catalytic site of P450 or by binding directly to the haem iron, thereby inhibiting electron flow to the xenobiotics (Goeptar et al 1995).

Several studies have described the inhibitory effects of 18β-glycyrrhetinic acid on steroid metabolising enzymes, such as 5α-reductase, 5β-reductase, 3α-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase (Akao et al 1992; Maser et al 1996), and have revealed that 18β-glycyrrhetinic acid potently inhibits the activity of these enzymes.

11β-Hydroxysteroid dehydrogenase (11β-HSD, EC 1.1.1.146) is a microsomal enzyme responsible for the interconversion of active 11-hydroxy-glucocorticoids to inactive 11-oxo forms (Monder & Shackleton 1984). It is generally accepted that there are at least two isoforms of 11β-HSD in mammals, a ubiquitous low-affinity $NADP^+$-dependent enzyme (11β-HSD 1) and a tissue-specific high-affinity NAD^+-dependent form (11β-

Correspondence: B. Szotáková, Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, CZ-500 05 Hradec Králové, Czech Republic.

HSD 2). One of the physiological functions of 11β-HSD 1 is its capability of catalysing the reductive metabolism of xenobiotic carbonyl compounds (Maser 1996; Oppermann et al 1997).

Carbonyl reduction of biologically and pharmacologically active xenobiotic carbonyl compounds to the corresponding alcohols is generally mediated by cytosolic NADPH-dependent enzymes. Cytosolic carbonyl reductase (EC 1.1.1.184) plays the most striking role in xenobiotic carbonyl reduction and is specifically inhibited by the diagnostic inhibitor quercitrin (Felsted & Bachur 1980; Wermuth 1985; Büld et al 1996; Pröpper & Maser 1997). Carbonyl reductase is widely distributed in human and other mammalian tissues, it catalyses the NADPH-dependent reduction of a variety of carbonyl compounds, for example quinones derived from polycyclic aromatic hydrocarbons, 9-keto-prostaglandins and the anthracycline antibiotic daunorubicin (Wermuth et al 1988).

Oracin (Figure 1), {6-[2-(2-hydroxyethyl)aminoethyl]-5,11-dioxo-5,6-dihydro-11*H*-indeno[1,2-*c*]isoquinoline}, is a new and potent cytostatic agent for oral use (Michalský 1992). Oracin significantly reduced the tumour weight of the ascites form of S37 sarcoma and of the solid form of Ehrlich carcinoma and HK test model adenocarcinoma. It also extended the life span of rats with Yoshida reticulosarcoma and mice with ascites S37 sarcoma, L1210 and P388 leukaemias, Gardner lymphosarcoma and the solid form of Ehrlich carcinoma (Mělka 1993). The main advantage of oracin is its negative result in the Ames test (Marhan 1995) and lack of cardiotoxicity (Geršl et al 1996).

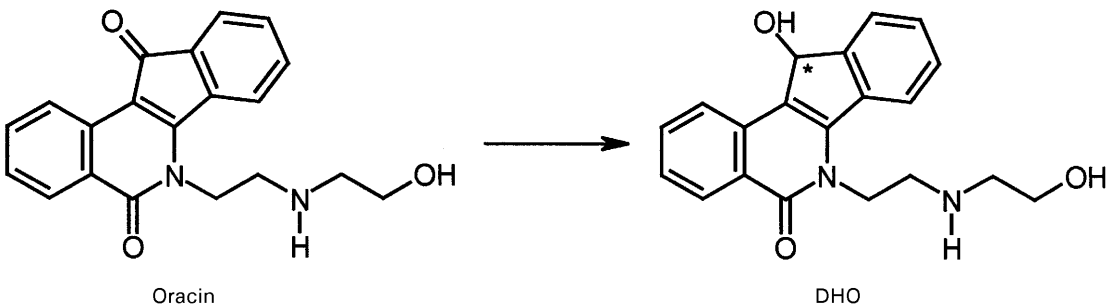
Due to its important biological activity and positive properties, biotransformation of oracin has been intensively studied. One of the main metabolites of oracin is 11-dihydrooracin (DHO), {6-[2-(2-hydroxyethyl)aminoethyl]-5-oxo-11-hydroxy-5,6-dihydro-11*H*-indeno[1,2-*c*]isoquinoline} (Figure 1), a metabolite formed by the reduction of oracin's prochiral centre on C 11. The studies were performed in-vitro (Szotáková et al 1996) and in-vivo (Wsól et al 1996; Szotáková et al 1998) using standard experimental animals. DHO arises from oracin during incubation with rat hepatic microsomal fraction more easily in anaerobic than in aerobic conditions and in a greater amount with coenzyme NADPH than with NADH. Cytosol also takes part in oracin reduction and a preference for NADPH coenzyme and anaerobic conditions was observed (Skálová et al 1999; Wsól et al 1999). DHO has been found in all species of laboratory animals studied and it constitutes the main metabolite in the urine and plasma of man (Wsól et al 1998). As indicated above, reduction of oracin leads to production of a chiral metabolite. The effect of animal species, and sex differences in the rat, on the stereospecificity of oracin reduction has been also studied (Wsól et al 1998, 1999; Skálová et al 1999).

Although constituting the major pathway of oracin metabolism, the enzymes which catalyse the carbonyl reduction of oracin to DHO in both microsomal and cytosolic fractions have not yet been identified. This study was designed to evaluate and compare the extent of oracin carbonyl reduction in microsomal and cytosolic fractions, and to identify the enzymes involved under these conditions.

Materials and Methods

Chemicals

Oracin was obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Coenzyme NADPH, inhibitors (metyrapone, α-naphthoflavone, quercitrin, methylpyrazole, 18β-glycyrrhetinic acid) and inducers (3-methylcholanthrene, phenobarbital) were purchased from Sigma-Aldrich (Prague, Czech Republic). Acetonitrile and methanol (both HPLC



Oracin DHO

Figure 1. Chemical structure of oracin and 11-dihydrooracin (DHO).

grade) were obtained from Merck (Prague, Czech Republic). Carbon monoxide was from Linde-Technoplyn (Prague, Czech Republic). All other chemicals were of the highest purity commercially available.

Animals and biological materials

Male Wistar rats (200–240 g, 8–10 weeks) were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). They were kept on standard rat chow with free access to tap water, in animal quarters under a 12-h light–dark cycle. Experiments were performed according to international guidelines. The liver homogenate was prepared in the ratio of 1 : 3 (w/v) in 0·1 M Na-phosphate buffer pH 7·4 in the Potter and Elvehjem homogeniser. Microsomal and cytosolic fractions were obtained by fractional ultracentrifugation of the liver homogenate in the same buffer (Gillette 1972).

Induction studies

Oral doses of phenobarbital were administered to the rats for 5 days (1 mL solution in drinking water, 60 mg kg^{-1} per day), oral doses of ethanol were administered for 10 days (1 mL 20% (v/v) solution in drinking water) and the last group of rats was pre-treated with intraperitoneal injection of 3-methylcholanthrene for 3 days (1 mL solution in corn oil, 25 mg kg^{-1} per day). The last dose was given 24 h before isolation of subcellular fractions. The control rats were given drinking water or corn oil only.

Protein content determination and enzyme assays

Protein was determined according to the modified method of Lowry with 0·1% SDS (Markwell et al 1978). The amount of P450 was estimated using the method of Omura & Sato (1964) of differential spectrum analysis after CO reduction. Aniline hydroxylase activity was assayed according to Mazel (1972) by measuring arising *p*-aminophenol. Ethoxyresorufin *O*-deethylase (EROD) and penthoxyresorufin *O*-dealkylase (PROD) activity assays were carried out according to Lubet et al (1985).

Inhibition experiments

The classic inhibitor of cytochrome P450, carbon monoxide, was used to evaluate the involvement of P450 in oracin reduction. Inhibition studies were performed by adding 1 μmol of a stock solution of α-naphthoflavone, metyrapone or methylpyrazole to the standard incubation mixture (see below). Glycyrrhetinic acid, a potent inhibitor of 11β-HSD, and quercitrin, the diagnostic inhibitor of carbonyl reductase, were used in final concentrations: 0–4 μM for glycyrrhetinic acid and 0–4000 μM for quercitrin.

Incubation and extraction

Standard incubation mixtures were prepared in Eppendorf microtubes. The microsomal suspension (0·1 mL containing 0·9–1·2 mg of protein in 0·1 M Na-phosphate buffer, pH 7·4, i.e. a sample corresponding to 0·1 g of the original wet liver tissue) or the cytosolic fraction (0·1 mL containing 0·9–1·2 mg of protein in 0·1 M Na-phosphate buffer, pH 7·4, i.e. a sample corresponding to 0·014 g of the original wet liver tissue) was incubated with 0·4 μmol of substrate and 0·6 μmol of coenzyme NADPH in a total buffer volume of 0·3 mL. Incubations at 37°C under aeration were performed for 30 min. All incubations were terminated by cooling to 0°C and adding 6% aqueous ammonia solution to pH 10·8–11·0, and extracted three times with two volumes of distilled ethyl acetate; combined extracts were evaporated to dryness under vacuum. The dry samples were dissolved in the mobile phase before injection onto the HPLC column.

High-performance liquid chromatography

Liquid chromatography was carried out using a Spectra Series P200 gradient pump from Spectra-Physics (Fremont, CA), a HP 1100 Series autosampler, a HP 1100 Series thermostatted column compartment from Hewlett Packard (Waldbronn, Germany) a multichannel UV-Vis detector PU4021 and a fluorescence detector PU4027 from Pye Unicam (Cambridge, England). DHO was detected using a detection wavelength of 340 nm (UV-Vis), an excitation wavelength of 340 nm and an emission wavelength of 418 nm (a more sensitive fluorescence detector). Data from chromatographic runs were processed using a chromatography station for Windows CSW (version 1.6) software from DataApex (Prague, Czech Republic) on a 486/66 PC from AutoCont (Hradec Králové, Czech Republic). The whole study was performed using a 250 × 4 mm C18 Spherisorb ODS2 (5 μm) column. The mobile phase was prepared by mixing buffer (10 mmol L^{-1} sodium 1-hexanesulphonate and 0·1 mol L^{-1} triethylamine; H_3PO_4 to pH 3·27) with acetonitrile (75 : 25 v/v). The temperature was

25°C and flow rate was 1·8 mL min^{-1} (Wsól et al 1996).

Results

Oracin reduction caused by cytochrome P450

Microsomal oracin reduction was first studied from the aspect of the cytochrome P450 contribution to the process. One of the most common approaches employed to identify the role of P450 in the biotransformation process is the use of inhibitors and inducers.

Carbon monoxide caused a significantly (Student's *t*-test for unpaired observation between control and experimental samples, $P < 0{\cdot}01$) higher yield of DHO and the same tendency was achieved in argon atmosphere (Table 1).

Among several inducers of rat cytochrome P450, only 3-methylcholanthrene, the selective inducer of P4501A, caused a significant ($P < 0{\cdot}01$) stimulation of DHO production by 190% in comparison with control microsomes. The relatively specific inhibitor of cytochrome P4501A in the rat, α-naphthoflavone, significantly ($P < 0{\cdot}01$) decreased this induced reduction activity to the level found in control microsomes (100%). Phenobarbital, a cytochrome P4502B inducer, had no effect on the formation of DHO but a statistically significant ($P < 0{\cdot}01$) inhibition was caused by metyrapone, an inhibitor of cytochrome P4502B in the rat. Metyrapone decreased oracin reduction by 95% ($P < 0{\cdot}01$) in comparison with samples incubated in the absence of inhibitors. Neither ethanol nor methylpyrazole had any effect on DHO production. Results are shown in Table 2.

The induction effect of 3-methylcholanthrene, phenobarbital and ethanol was confirmed by assays of EROD, PROD and aniline hydroxylase activity, respectively. The EROD activity in 3-methylcholanthrene-treated microsomes was induced by a factor of 350, the PROD activity in phenobarbital treated rats was 55 times higher and the aniline hydroxylase activity was raised by 130% (Szotáková et al 1999).

Table 1. The effect of carbon monoxide on the microsomal reduction of oracin under aerobic or anaerobic conditions.

Treatment	Activity (nmol DHO (30 min)$^{-1}$ (mg protein)$^{-1}$)
Microsomes (aerobic)	0·482 ± 0·025
Microsomes + CO (aerobic)	0·582 ± 0·040*
Microsomes (anaerobic)	0·808 ± 0·014*
Microsomes + CO (anaerobic)	1·005 ± 0·028*

Values are presented as mean ± s.d. of six samples; *$P < 0{\cdot}01$ compared with microsomes (aerobic).

Table 2. The effect of inducers (phenobarbital, 3-methylcholanthrene, ethanol) and inhibitors (metyrapone, α-naphthoflavone, methylpyrazole) on rat-liver microsomal reduction of oracin.

Treatment	Activity (nmol DHO (30 min)$^{-1}$ (mg protein)$^{-1}$)
Control	0·485 ± 0·030
Control + metyrapone	0·029 ± 0·006*
Phenobarbital	0·445 ± 0·050
Phenobarbital + metyrapone	0·024 ± 0·006*
3-Methylcholanthrene	1·407 ± 0·129*
3-Methylcholanthrene + α-naphthoflavone	0·460 ± 0·027
Ethanol	0·407 ± 0·065
Ethanol + methylpyrazole	0·330 ± 0·076

Values are expressed as mean ± s.d. of six samples; *$P < 0{\cdot}01$ compared with control.

Inhibitory effect of 18β-glycyrrhetinic acid on microsomal HSD reduction of oracin

Microsomal HSD was inhibited by 18β-glycyrrhetinic acid. Table 3 illustrates that 18β-glycyrrhetinic acid is a potent inhibitor of oracin carbonyl reduction. The amount of DHO was significantly ($P < 0{\cdot}01$) lower after incubation of the rat hepatic microsomal fraction with 18β-glycyrrhetinic acid than in the control samples. In rat liver microsomes, 50% inhibition was already achieved at a glycyrrhetinic acid concentration of 0·15 μM.

Inhibition of cytosolic oracin reduction

Participation of rat cytosolic carbonyl reductase was investigated. Quercitrin was used to identify the cytosolic enzyme(s) responsible for oracin reduction at physiological pH 7·4. As shown in Table 4, quercitrin displays a significant ($P < 0{\cdot}01$) inhibitory effect on the reduction of oracin to DHO. In rat liver cytosol, 50% inhibition was obtained at a quercitrin concentration of 260 μM.

Table 3. The inhibitory effect of 18β-glycyrrhetinic acid on oracin carbonyl reduction in rat liver microsomes.

18β-Glycyrrhetinic acid concn (μM)	Activity (nmol DHO (30 min)$^{-1}$ (mg protein)$^{-1}$)
0·00	0·477 ± 0·054
0·10	0·284 ± 0·021*
0·25	0·145 ± 0·017*
0·50	0·076 ± 0·034*
1·00	0·049 ± 0·014*
2·00	0·027 ± 0·012*
4·00	0·016 ± 0·008*

Each value represents the mean ± s.d. of 8 rats; *$P < 0{\cdot}01$ compared with non-inhibited reaction.

Table 4. The inhibitory effect of quercitrin on oracin carbonyl reduction in rat-liver cytosol.

Quercitrin concn (μM)	Activity (nmol DHO $(30\,min)^{-1}$ $(mg\ protein)^{-1}$)
0·0	0·364 ± 0·062
0·8	0·357 ± 0·077
8·0	0·303 ± 0·041
80·0	0·222 ± 0·029*
800·0	0·056 ± 0·014*
4000·0	0·001 ± 0·001*

Each value represents the mean ± s.d. of 8 rats; $*P < 0{\cdot}01$ compared with non-inhibited reaction.

Discussion

As cytochrome P450 and its reductase play an important role in the reduction of some xenobiotics bearing a carbonyl group (Goeptar et al 1995), participation of cytochrome P450 in oracin reduction was considered. The influence of oxygen on oracin microsomal reduction (NADPH dependent) was also determined because oxygen is thought to be an important inhibitor of the xenobiotic reductase activity of cytochrome P450 and a significant increase in DHO production under anaerobic conditions was observed. To evaluate the involvement of cytochrome P450 in oracin reduction, carbon monoxide was used (Ortiz de Montellano & Reich 1986). The binding of carbon monoxide to cytochrome P450, surprisingly, significantly ($P < 0{\cdot}01$) increased the yield of DHO. This observed increase in DHO formation could be explained by inhibiting the reoxidation of DHO to oracin (Skálová et al 1999), which is probably catalysed by cytochrome P450. Carbon monoxide binding inhibits the oxidation activity of cytochrome P450 but its reductase activity is favoured.

Among several inducers of rat cytochrome P450, only 3-methylcholanthrene caused a significant stimulation of DHO production and the inhibitor of cytochrome P4501A, α-naphthoflavone, significantly ($P < 0{\cdot}01$) decreased induced reduction activity. It can be concluded from these findings that participation of cytochrome P450 in oracin reduction is important only after P4501A induction.

The finding that cytochrome P450 plays only an inessential role in oracin reduction prompted the search for another NADPH-dependent enzyme taking part in this reduction in microsomal fraction of the rat liver. 18β-Glycyrrhetinic acid, the inhibitor of hydroxysteroid dehydrogenase (HSD), was used to evaluate the involvement of HSD in the reduction of oracin. The production of DHO was significantly ($P < 0{\cdot}01$) lower under these incubation conditions (microsomal fraction, oracin and 18β-glycyrrhetinic acid) in comparison with the non-inhibited reaction. The steroidal compound 18β-glycyrrhetinic acid is a potent inhibitor of oracin carbonyl reduction (>95% inhibition, $IC50 = 0{\cdot}15\ \mu M$). These findings correspond well to those of this diagnostic HSD inhibitor obtained in other studies (Akao et al 1992; Maser et al 1996). As can be seen from the chemical structure of cortisone and 11-dehydrocorticosterone, the position of carbonyl group (C 11) is very similar to that of oracin which also has a carbonyl group on C11 (of the indenoisoquinoline skeleton). From this aspect oracin seems to be a good substrate for 11β-hydroxysteroid dehydrogenase (11β-HSD). The participation of 11β-HSD in oracin reduction was confirmed by the use of metyrapone, which not only inhibits cytochrome P4502B in the rat (Souček & Gut 1992) but is also the substrate of 11β-HSD (Maser & Bannenberg 1994), and indeed it caused a statistically significant ($P < 0{\cdot}01$) inhibition (95%) of DHO production. In this study the authors found that the main microsomal enzyme which catalyses the carbonyl reduction of oracin is probably 11β-HSD.

Cytosol also takes part in oracin reduction and preference for NADPH coenzyme and anaerobic conditions was observed (Skálová et al 1999 ; Wsól et al 1999). At first glance the cytosolic reduction seems to be lower (the amount of DHO per mg of protein is lower than in microsomes) but when the DHO production is counted per mg of the wet liver tissue the reduction in the cytosolic fraction is higher (approximately 7 times). According to its sensitivity to quercitrin (99% inhibition, $IC50 = 260\ \mu M$) (Pröpper & Maser 1997), the enzyme responsible for DHO formation in rat liver cytosol seems to be carbonyl reductase.

The potential anticancer drug oracin is reduced to DHO in both rat liver microsomes and cytosol. The enzymes taking part in oracin reduction were characterised in both subcellular fractions. The participation of several enzymes in the main metabolic transformation of oracin is (from the pharmacological point of view) an advantageous property of this drug, for the function of one (e.g. genetically missing or inhibited) enzyme can be replaced by another one. The biotransformation of such a substrate is also less sensitive towards drug–drug interactions and smaller interindividual differences in biotransformation could be expected.

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