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Effect of the anticancer drug oracin on mouse liver topoisomerases I and II

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Abstract

The inhibitory effect of oracin on the activities of topoisomerases I and II isolated from the nuclei of mice liver tissue was studied. This drug showed a 100% inhibitory effect at 5 μM and 50% inhibition at 1 μM on topoisomerase II activity, while the activity of topoisomerase I at these concentrations was not inhibited.

Introduction

Oracin is a novel anticancer agent of the indenoisoquinoline chemical group (Melka 1994) and it is both structurally and pharmacologically related to the clinically used anticancer agent mitoxantrone, which is an anthracenedione anti-neoplastic agent and a cleavage-inducing inhibitor of topoisomerase II (O'Reilly & Kreuzer 2002).

Oracin revealed direct cytotoxicity against a wide spectrum of tumour and leukaemia cells in-vitro and in-vivo. The planar fundamental structure of the compound is typical for intercalating antitumour agents (anthracyclines, e.g. adriamycin-like drugs, and anthracenediones, e.g. mitoxantrone-like agents). The main advantage of oracin is its lack of cardiotoxicity – a dose-limiting adverse effect of doxorubicin-like drugs and possibly of its oral administration (Szotáková et al 2000; Miko et al 2002).

The cytotoxic effect of the new potential anticancer drug oracin has been studied on Burkitt's lymphoma (BL) cells with over-expression of the bcl-2 gene and BL SV2 cells (containing control vector). Expression of the survival gene-like bcl-2 in cancer cells is thought to be an impediment to genotoxic cancer therapy and could provide protection against apoptosis induced by drugs that interfere with DNA replication of cancer cells (Cory 1995; Fisher et al 1995; Klučar & Al-Rubeau 1997).

Metabolic studies on oracin have revealed that the principal metabolite in all laboratory animals is 11-dihydrooracin, which is produced by carbonyl reduction of the parent compound. Phase II of the clinical evaluation of oracin is ongoing (Szotáková et al 2003; Wsól et al 2003).

Topoisomerases are essential enzymes that play a role in virtually every cellular DNA process and thus are the target for some of the most successful anticancer drugs used to treat human malignancies. These agents are referred to in the case of topoisomerase II as poisons because they transform the enzyme into a potent cellular toxin. Topoisomerases I and II are also the target for a second category of drugs referred to as catalytic inhibitors. Compounds in this category prevent topoisomerases from carrying out their required physiological functions (Wo et al 1999; Fortune & Osheroff 2000).

In the present work the effect of oracin on the activities of topoisomerases I (EC 5.99.1.2.) and II (EC 5.99.1.3.), which were isolated from the nuclei of mice liver cells, was studied.

Material and Methods

Chemicals

Chromatographically pure oracin was from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). The substance was dissolved in DMSO immediately before use as stock solution. All other chemicals were of analytical grade.

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Biological materials

The liver tissue (a kind gift from the Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia) that was used as a source of nuclear topoisomerases was prepared from a 6-week-old male of the Male H Swiss albino mouse strain.

Topoisomerase isolation from the nuclei of mouse liver tissue

Topoisomerases I and II were isolated from nuclei isolated from the mouse liver according to Miller et al (1981) and Miko et al (2000) with the following modifications. The liver tissue was cooled to 4°C and the whole procedure was performed at a temperature between 0 and 4°C. The tissue (5.6 g wet weight) was centrifuged ($3000 \times g$, 10 min), washed twice with 25 mL of washing buffer (0.15 M NaCl, 10 mM sodium phosphate, pH 7.5) and then resuspended in 25 mL of extraction buffer (5 mM potassium phosphate, pH 7.0, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) added as 100 mM solution in 2-propanol, 1 mM 2-mercaptoethanol (2-ME), 0.5 mM 1,4-dithiothreitol (DTT) and 0.1 mM EDTA). After incubation at 0°C for 30 min the cells were thoroughly homogenized by a Potter homogenizer. The nuclei were harvested by centrifugation ($200 \times g$ for 10 min) and then washed with 13 mL of nuclei washing buffer (extraction buffer without $MgCl_2$ and EDTA).

Purification of topoisomerases I and II from nuclei

Preparation of so-called polyethylene glycol (PEG) supernatant

Isolated nuclei were resuspended in 25 mL of nuclei washing buffer and EDTA was added to the final concentration of 4 mM. After 15 min at 0°C the nuclei were lysed while stirring by slow addition of 25 mL of buffer containing 2 M NaCl, 50 mM TrisHCl pH 7.5, 10 mM 2-ME and 1 mM PMSF. Then 25 mL of 18% (w/v) PEG, 1 M NaCl, 50 mM TrisHCl pH 7.5, 10 mM 2-ME and 1 mM PMSF was added slowly after another 15 min at 0°C while stirring. This solution was incubated for 40 min at 0°C with occasional stirring and then centrifuged at $12\,000 \times g$ for 30 min. Supernatant fraction I (65 mL) was used for the following purification.

Column chromatography on hydroxylapatite

Thirty millilitres of fraction I was loaded onto a column with hydroxylapatite (1.5×15 cm) equilibrated with buffer containing 6% PEG, 1 M NaCl, 50 mM TrisHCl pH 7.7, 10 mM 2-ME and 1 mM PMSF. The column was washed with 20 mL of 0.2 M potassium phosphate pH 7.0 in buffer A (10% glycerol, 10 mM 2-ME, 1 mM PMSF) and gradient 0.2–0.7 M potassium phosphate pH 7.0 in buffer A was applied onto the column for protein elution. Topoisomerase II (fraction II) was eluted from the column slightly before topoisomerase I.

Determination of topoisomerase I activity

The activity was measured in 20 μL of reaction mixture TOPO-I (50 mM TrisHCl pH 7.9, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 $\mu g\,mL^{-1}$ bovine serum albumin (BSA), 20 $\mu g\,mL^{-1}$ DNA (pUC21)). Enzyme sample (1 or a maximum of 2 μL) was added to the reaction and the mixture was incubated for 30 min at 30°C. The reaction was stopped by adding 5 μL 5% SDS, 25% Ficoll and 0.25 mg mL^{-1} bromophenol blue. Reactions were analysed on 1% horizontal agarose gel (3 h at 6 V cm^{-1}) and the activity of the enzymes was determined after DNA staining by ethidium bromide.

Determination of topoisomerase II activity

The activity was measured in 20 μL of reaction mixture TOPO-II (50 mM TrisHCl pH 7.9, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 $\mu g\,mL^{-1}$ BSA, 0.5 mM ATP and 20 $\mu g\,mL^{-1}$ DNA (P4 DNA was used for the decatenation reaction and plasmid pUC21 was used for the relaxation reaction). The reactions were stopped after incubation for 30 min at 30°C by adding 5 μL 5% SDS, 25% Ficoll and 0.25 mg mL^{-1} bromophenol blue. Reactions were analysed on 1% horizontal agarose gel (3 h at 6 V cm^{-1}). The definition of one enzyme unit is the amount of topoisomerase II that relaxes 50% of plasmid DNA (or decatenates 50% of P4 DNA) under the above conditions.

Determination of the oracin inhibition effect on the activity of topoisomerases

The stock solution of oracin (100 mM) was prepared in DMSO. The inhibition effect was determined by electrophoresis of the reaction in 1% agarose gel. Digitalized electrophoretographs were analysed by Image Quant software (Molecular Dynamics) and the percentage of integrated peak area of superhelical plasmid form covalently closed circular (CCC) was taken as a percentage of inhibition.

Results and Discussion

DNA topoisomerase activity was determined in the nuclei lysate of mouse liver cells. Knotted P4 DNA was used to separate the topoisomerase II activity from the activity of topoisomerase I (data not shown). Both topoisomerases were then purified by column chromatography for the inhibition experiments. All topoisomerase I experiments were performed in a reaction mixture without ATP therefore possible contamination by topoisomerase II, and thus its interference activity in this assay, was excluded because topoisomerase II is totally inactive in such conditions.

Time-dependent kinetic analysis (10, 30 and 60 min reactions) of topoisomerase I inhibition was performed (Figure 1A). The result of this experiment showed that oracin at a concentration of 30 μM revealed a strong intercalation effect and therefore precise values of possible topoisomerase I inhibition at the higher concentrations could not be determined by this method. We found that oracin in-vitro

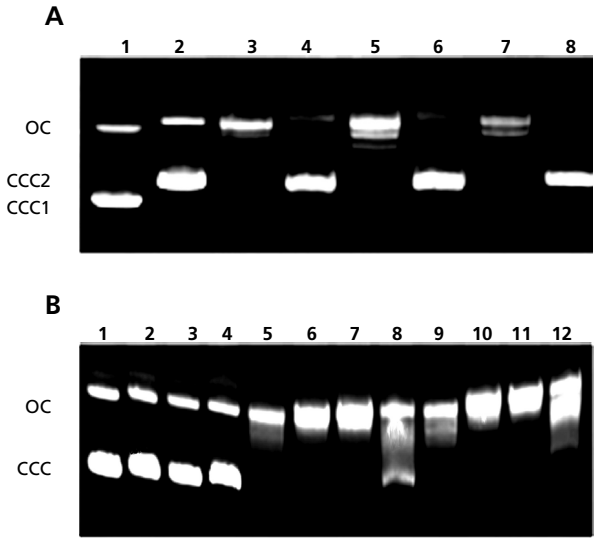

Figure 1 (A) Time-dependent kinetics of topoisomerase I inhibition with 30 μM oracin. CCC1, covalently closed circular DNA plasmid form in absence of oracin; CCC2, covalently closed circular DNA plasmid form in the presence of 30 μM oracin (shift of CCC1 into CCC2 is caused by intercalation effect of oracin); OC, open circular DNA plasmid form. Lanes: 1, pUC21; 2, pUC21 + 30 μM oracin; 3, pUC21 + topoisomerase I (10 min reaction); 4, pUC21 + 30 μM oracin + topoisomerase I (10 min reaction); 5, pUC21 + topoisomerase I (30 min reaction); 6, pUC21 + 30 μM oracin + topoisomerase I (30 min reaction); 7, pUC21 + topoisomerase I (60 min reaction); 8, pUC21 + 30 μM oracin + topoisomerase I (60 min reaction). (B) Kinetics of topoisomerase II inhibition with oracin concentrations of 1 and 5 μM. Lanes: 1, pUC21; 2, pUC21 + 0.1 μM oracin; 3, pUC21 + 1 μM oracin; 4, pUC21 + 5 μM oracin; 5, pUC21 + topoisomerase II (10 min reaction); 6, pUC21 + 0.1 μM oracin + topoisomerase II (10 min reaction); 7, pUC21 + 1 μM oracin + topoisomerase II (10 min reaction); 8, pUC21 + 5 μM oracin + topoisomerase II (10 min reaction); 9, pUC21 + topoisomerase II (30 min reaction); 10, pUC21 + 0.1 μM oracin + topoisomerase II (30 min reaction); 11, pUC21 + 1 μM oracin + topoisomerase II (30 min reaction); 12, pUC21 + 5 μM oracin + topoisomerase II (30 min reaction).

inhibited up to 30% of the activity of topoisomerase I at a concentration of 15 μM (data not shown).

It was necessary to exclude the interference of topoisomerase I when the inhibitory effect on topoisomerase II was determined. After analysis of 60 min of incubation in a reaction mixture without ATP only minimal relaxation of CCC plasmid DNA form occurred (data not shown).

Topoisomerase II activity was inhibited by up to 50% by oracin at a concentration of 1 μM (Figure 1B). It is evident from the results of this study that the main target of oracin biological activity is inhibition of topoisomerase II, but at concentrations higher than 15 μM it also inhibits topoisomerase I. An overview of oracin inhibitory properties on the activity of topoisomerases I and II is shown in Table 1.

Conclusions

The results show that the main target of oracin (like other pharmacologically related drugs) is topoisomerase II. Topoisomerase I inhibition probably does not play an important role in the oracin effect because its activity is not affected at the inhibitory oracin concentration for topoisomerase II.

Table 1 Inhibitory effects of oracin on topoisomerase I and II activities

Enzyme	Inhibition (%)[a]			
	0.1 μM	1 μM	5 μM	15 μM
Topoisomerase I	0	0	0	30
Topoisomerase II	0	50	100	100

[a]The data in this table were determined from five independent experiments. The standard deviation values (σ) were calculated by the biased method and were in intervals of 4.9 to 5.5.

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