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Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes

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Abstract

Ellipticine is a potent antineoplastic agent whose mechanism of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II. Recently, we found that ellipticine also forms covalent DNA adducts and that the formation of the major adduct is dependent on the activation of ellipticine by cytochrome P450 (CYP). We examined a panel of genetically engineered V79 cell lines including the parental line V79MZ and recombinant cells expressing the human CYP enzymes CYP1A1, CYP1A2 or CYP3A4 for their ability to activate ellipticine. The extent of activation was determined by analysing DNA adducts by ^{32}P-postlabelling. Ellipticine was found to be toxic to all V79 cell lines with IC_{50} values ranging from 0.25 to 0.40 μM. The nuclease P1 version of the ^{32}P-postlabelling assay yielded a similar pattern of ellipticine-DNA adducts with two major adducts in all cells, the formation of only one of which was dependent on CYP activity. This pattern is identical to that detected in DNA reacted with ellipticine and the reconstituted CYP enzyme system *in vitro* as confirmed by HPLC of the isolated adducts. Total adduct levels ranged from 2 to 337 adducts per 10^8 nucleotides, in the parental line and in V79 expressing CYP3A4, respectively. As *in vitro*, human CYP1A2 and CYP1A1 were less active. The results presented here are the first report showing the formation of CYP-mediated covalent DNA adducts by ellipticine in cells in culture, and confirm the formation of covalent DNA adducts as a new mechanism of ellipticine action. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: ^{32}P-Postlabelling; Calcein-assay; V79 Chinese hamster fibroblasts; Human cytochrome P450

1. Introduction

Ellipticine (5,11-dimethyl-6-*H*-pyrido[4,3-*b*]carbazole) an alkaloid isolated from *Apocyanaceae* plants and several of its more soluble derivatives (9-hydroxyellipticine, 2*N*-methyl-9-hydroxyellipticinium, 2*N*-methyl-9-chloroellipticinium and 2*N*-methyl-9-methoxyellipticinium) exhibit significant antitumour and anti-HIV activities (for a summary see [1]). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects and their complete lack of haematological toxicity [2].

Most ellipticines are mutagenic to *Salmonella typhimurium* Ames tester strains, bacteriophage T4, *Neurospora crassa*, and mammalian cells and induce prophage lambda in *Escherichia coli* (for an overview see [1]).

Ellipticines are anticancer drugs, whose precise mechanisms of action have not yet been explained. It was suggested that the prevalent mechanisms of ellipticine antitumour activities are (i) intercalation into DNA [3,4] and (ii) inhibition of DNA topoisomerase II activity [2,5–7]. Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines [8], and this correlated with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation [9], and thereby disrupt the energy balance of cells.

Recently, we found that ellipticine covalently binds to DNA after being enzymatically activated. The cytochrome P450 (CYP) isoenzymes known to be expressed at higher levels in tumours sensitive to ellipticine (i.e. breast cancer,

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Abbreviations: CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; IC_{50}, 50% inhibitory concentration; PEI, polyethylenimine; V79MZ, parental Chinese hamster fibroblast cell line; V79MZ-h1A1, V79MZ-h1A2 and V79MZ-h3A4, recombinant V79MZ cells expressing human CYP1A1, CYP1A2 and CYP3A4, respectively.

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renal cell cancer) than in peritumoural tissues, namely CYP3A4, CYP1A1 and CYP1B1 [10–12] were the most efficient in activating ellipticine to form covalent DNA adducts *in vitro* [1]. Hence, ellipticine might be considered a pro-drug, whose pharmacological efficiency is dependent on its enzymatic activation in target tissues.

Here, we examine whether ellipticine is activated in cells transfected with CYP-enzymes, and if so which DNA adducts are formed in this cellular system as an intermediate between our *in vitro* system and the study with animals. V79 Chinese hamster lung fibroblasts are easy to handle but completely lack CYP enzymes [13]. V79 cells were therefore transfected with several human CYP enzymes and found to be appropriate models to study the activation of xenobiotics by specific CYP [13]. A panel of genetically engineered V79 cell lines expressing human CYP1A1, CYP1A2 or CYP3A4 including the parental cell line V79MZ were employed in our work. The ^{32}P-postlabelling method to determine DNA adduct formation by ellipticine was used and cytotoxicity of ellipticine was determined with the calcein assay [14].

2. Material and methods

2.1. Chemicals and cell lines

Calcein-AM glycine (*N*,*N*′-[[3′,6′-bis(acetyloxy)-3-oxospiro[isobenzfuron-1(3*H*),9′-[9*H*]xanthene]-2′,7′-diyl]bis-(methylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-bis[(acetyloxy)methyl] ester) was purchased from MoBiTec. Ellipticine was obtained from Sigma.

The recombinant cell lines V79MZ-h1A1 expressing human CYP1A1, V79MZ-h1A2 expressing human CYP1A2 and V79MZ-h3A4 expressing human CYP3A4 in conjunction with human NADPH:CYP reductase [13] were purchased from PharmBioDyn. Coexpression of human CYP3A4 and NADPH:CYP reductase has been reported to be needed for higher CYP3A4 activity [15]. The parental cell line V79MZ was kindly provided by Doehmer (GenPharmTox BioTech AG). According to the manufacturers suggestions cells were used between passages 3 and 10 to ensure effective CYP expression.

2.2. Cell culture and protein analysis

The V79 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Biochrom KG), high-glucose type (DMEM with 4.5 g D-glucose/L), supplemented with 1 mM sodium pyruvate (Biochrom KG), 4 mM L-glutamine (PAA Laboratories), 25 mM HEPES (Gibco), 5% foetal calf serum (Biochrom KG), 100 U penicillin/mL and 100 μg streptomycin/mL (Biochrom KG) at 37°, 5% CO_2 and 95% saturated atmospheric humidity.

The presence of human CYP1A1, CYP1A2 and CYP3A4 proteins in the recombinant V79 cell lines was screened in microsomal preparations by Western blot analyses utilising anti-human CYP1A1, CYP1A2 or CYP3A4 rabbit antiserum as primary antibodies and horseradish peroxidase-labelled pig anti-rabbit immunoglobulins as second antibodies (Gentest Corp.). Detection was with the enhanced chemoluminescence detection kit (Amersham).

2.3. Treatment of V79 cells with ellipticine for DNA adduct analyses

All V79 cells were seeded 24 hr prior to treatment at a density of 1×10^5 cells/mL in 75 cm^2 culture flasks in a total volume of 30 mL of DMEM. Ellipticine was dissolved in 30 μL dimethylsulphoxide (DMSO) and added to a final concentration of 1 μM. As control for enzyme activity the V79MZ, V79MZ-h1A1 and V79MZ-h1A2 lines were incubated for 24 hr with 1 or 10 μM benzo[*a*]-pyrene, then DNA was isolated and labelled as described in the next section. After the incubation periods indicated in Fig. 2 the medium was removed and the cells were washed twice with 5 mL PBS. During this step all dead cells were washed away. Cells were harvested by trypsinisation with 2 mL of a solution containing 0.025% trypsin and 0.01% EDTA (Biochrom KG) in PBS. Trypsinisation was stopped by addition of two times 4 mL of medium. Subsequently, centrifugation at 2000 rpm for 10 min and one washing step with 10 mL PBS yielded a cell pellet, which was stored at −20° until DNA isolation.

2.4. DNA isolation and ^{32}P-postlabelling of DNA adducts

DNA from cells was isolated by the phenol extraction version as described [16]. ^{32}P-Postlabelling analyses were performed using nuclease P1 enrichment as described previously [1]. In addition the standard procedure of the ^{32}P-postlabelling assay and the version of this technique using the 1-butanol extraction were also used. Separation was carried out on PEI-cellulose thin-layer plates (Macherey and Nagel). Chromatographic conditions used were: D1, 1.0 M sodium phosphate, pH 6.8; D3, 3.5 M Li-formiate, 8.5 M urea, pH 4.0; D4, 0.8 M LiCl, 0.5 M Tris, 8.5 M urea, pH 9.0; D5, 1.7 M sodium phosphate, pH 6.0. Normal nucleotides were separated in 280 mM $(NH_4)_2SO_4$, 50 mM NaH_2PO_4, pH 6.5. Quantitative analysis was performed using the Canberra Packard Instant Imager. Relative adduct labelling was calculated as cpm adducts per cpm unmodified nucleotides.

2.5. Co-chromatography on PEI-cellulose TLC

Adduct spots detected by the ^{32}P-postlabelling assay in DNA of V79 cells exposed to ellipticine and those in DNA incubated with ellipticine and human recombinant CYP3A4 *in vitro* [1] were excised from thin-layer plates, extracted, mixed and run on TLC plates as reported previously [17].

2.6. HPLC analysis of ^{32}P-labelled of adducts

HPLC analysis was performed essentially as described previously [17,18]. Individual spots detected by ^{32}P-post-labelling were excised from thin-layer plates and extracted [17]. The dried extracts were dissolved in 100 μL of methanol/phosphate buffer (pH 3.5) 1:1 (v/v). Aliquots (50 μL) were analysed on a phenyl-modified reversed-phase column (250 mm × 4.6 mm, 5 μm Zorbax Phenyl; Säulentechnik Knauer) with a linear gradient of methanol (from 40 to 80% in 45 min) in aqueous 0.5 M sodium phosphate and 0.5 M phosphoric acid (pH 3.5) at a flow rate of 0.9 mL/min. Radioactivity eluting from the column was measured by monitoring Cerenkov radiation with a Berthold LB 506 C-I flow through radioactivity monitor (500 μL cell, dwell time 6 s).

2.7. Calcein assay

The cytotoxicity of ellipticine was determined in a 96-well plate with the calcein assay. This is a fluorimetric assay based on the ability of live but not dead cells to hydrolyse the acetoxymethyl (AM) ester of calcein-AM to calcein which is fluorescent [14]. For a dose–response curve cells in exponential growth were seeded in 200 μL medium with 1000–1500 cells per well, 24 hr after seeding 100 μL of medium was replaced by medium containing ellipticine resulting in final concentrations of 0.05, 0.1, 0.5, 0.75, 1.0 and 2.0 μM. Control cells and medium controls without cells received 100 μL of medium without ellipticine. After an incubation period of 48 hr 100 μL of supernatant medium was replaced by 100 μL PBS containing calcein-AM yielding a final concentration of 10 μM and incubated for a further 2 hr at 37°. The optimal calcein-AM concentration and incubation time for V79 cells was determined beforehand. Fluorescence measurement was performed at 530 nm emission on a multi-plate reader (CytoFluor, PerSeptive Biosystems) with an excitation wavelength of 485 nm. The mean fluorescence of medium controls was the background and deducted. The fluorescence of control cells was taken as 100% viability and the values of treated cells were calculated as percent of control. Each value is the mean of 8 wells with SD. The IC_{50} values were calculated from the linear regression of the dose–log response curves.

3. Results

3.1. Determination of DNA adduct formation by ellipticine in V79 cell lines by ^{32}P-postlabelling

Western blot analyses showed the cells to express the CYP species desired, the parental V79MZ cells showed very faint traces of CYP3A4. The control incubations with benzo[*a*]pyrene showed the typical DNA adducts in the V79MZ-h1A1 cells and about 100-fold lower DNA adduct levels in the V79MZ-h1A2 cells as expected (data not shown).

V79 cell lines expressing human CYP1A1, CYP1A2 or CYP3A4 as well as parental cells were treated with 1 μM ellipticine for different time periods. Using the nuclease P1 version of the ^{32}P-postlabelling assay, ellipticine-derived adducts were detected in DNA of the parental cell line V79MZ as well as in that of the recombinant cell lines, V79MZ-h1A1, V79MZ-h1A2 and V79MZ-h3A4 (Fig. 1). The pattern of DNA adducts in each cell line consisted of two major spots after treatment with ellipticine (spots 1 and 2). The intensities of these spots were similar in the parental V79 line but spot 1 markedly increased in the cells expressing CYP, while spot 2 was unchanged. In V79 cells expressing human CYP3A4 exposed to ellipticine for more than 24 hr, two additional adduct spots, which were missing in other cells, were detected (data not shown). If the standard procedure of the ^{32}P-postlabelling assay or the

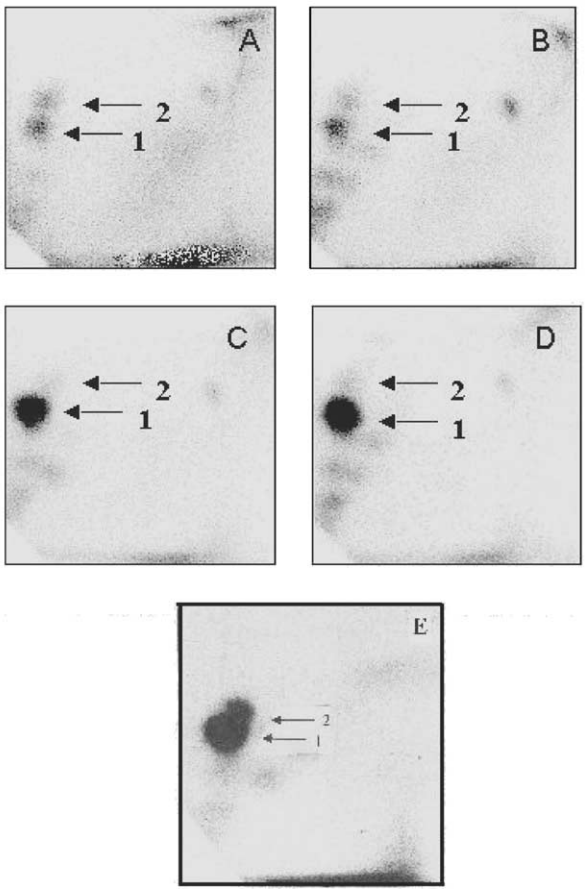

Fig. 1. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labelled digests of DNA isolated from (A) V79MZ, (B) V79MZ-h1A1, (C) V79MZ-h1A2, (D) V79MZ-h3A4 cells exposed to 1 μM ellipticine for 24 hr and (E) calf thymus DNA reacted with ellipticine, NADPH and recombinant human CYP3A4 reconstituted with NADPH:CYP reductase *in vitro* [1]. Analyses were performed by the nuclease P1 version of the ^{32}P-postlabelling assay. (A–D) Scans from the imager; (E) a film exposed for 2 hr at −80°. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

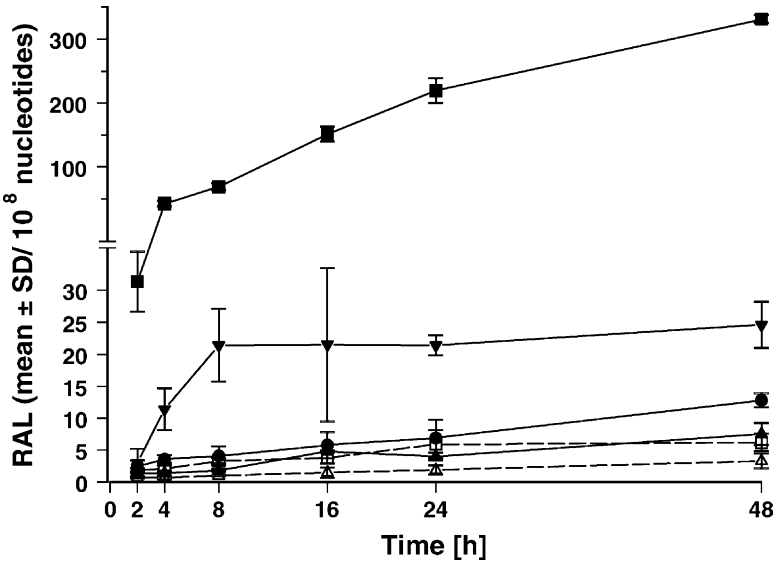

Fig. 2. Time dependence of ellipticine-DNA adduct levels formed in (▲) V79MZ adduct 1, (△) adduct 2, (●) V79MZ-h1A1, (▼) V79MZ-h1A2, (■) V79MZ-h3A4 adduct 1 and (□) adduct 2 in cells exposed to 1 μM ellipticine. For clarity adduct 2 levels (dotted lines) are only shown in V79MZ and V79MZ-h3A4 cells (RAL: relative adduct levels).

enrichment version using 1-butanol extraction of adducts [1] were used instead of the nuclease P1 enhancement, no differences in patterns and levels of ellipticine-DNA adducts were seen. No adducts were detected in DNA of control cells treated with solvent (DMSO) only (data not shown). For comparison Fig. 1E shows adducts in DNA reacted with ellipticine and human CYP3A4 reconstituted with NADPH:CYP reductase from experiments performed for our previous report [1].

Quantitative analyses, shown in Fig. 2, revealed a time-dependent formation of ellipticine-DNA adducts. The adduct levels were in a range from 2 to 337 adducts per 10^8 nucleotides for total DNA binding, being highest in V79 cells expressing human CYP3A4 and lowest in the parental V79MZ cells. The increase was solely in adduct spot 1 in recombinant V79 cells, adduct spot 2 levels were practically unchanged. The amount of adduct spot 1 was more than 44-fold higher in DNA of V79 cells expressing human CYP3A4 than in that of the parental cells after 48 hr. Furthermore, DNA adduct levels were significantly higher, 1.7-fold ($P = 0.05$) and 3.3-fold ($P = 0.005$) in V79 cells expressing human CYP1A1 and CYP1A2, respectively, than in V79MZ cells (Fig. 2). Human CYP1A1/2 are therefore also involved in the bioactivation of ellipticine in cells.

The pattern of spots was similar to that of ellipticine-derived DNA adducts found previously after *in vitro* incubation of calf thymus DNA with ellipticine and isolated CYP [1] (Fig. 1E). The adduct spots 1 and 2 obtained from DNA of V79 cells (V79MZ-h3A4) and those obtained from experiments with human recombinant CYP3A4 *in vitro* were excised, extracted and analysed by co-chromatography on PEI-cellulose TLC plates in directions D3 and D4. These experiments showed that the ^{32}P-labelled ellipticine adducts were stable under the alkaline extraction conditions used and that both major adducts in DNA of V79 cells were indistinguishable from those obtained in the *in vitro* experiments (data not shown).

As a second, independent chromatographic procedure to confirm identities of adduct spots we employed reversed-phase HPLC analyses. Individual spots were isolated from the PEI-cellulose plates and subjected to HPLC (Fig. 3). These results confirmed the co-chromatography on TLC. The major product (spot 1 in Fig. 1D) eluted with a retention time (rt) of 12.2 min, very similar to the rt of 11.68 min of adduct spot 1 in DNA exposed to ellipticine activated with purified human CYP3A4 *in vitro* (Fig. 3A and C). A minor peak of radioactivity was eluted at 6.22 min, showing that a minor adduct is present in cellular DNA comigrating with adduct spot 1 on TLC. Spot 2 of Fig. 1D produced three peaks of radioactivity, the major one (rt 9.07 min) corresponding to adduct spot 2 formed by ellipticine activated with purified human CYP3A4 *in vitro* (Fig. 3B and D). The additional peak with a rt of 12.03 min is a contamination of adduct spot 2 with adduct spot 1. The minor peak of radioactivity eluting at 14.78 min was not observed upon activation of ellipticine with CYP3A4 *in vitro*.

3.2. Cytotoxicity of ellipticine in V79 derived cell lines

To determine if the cytotoxicity of ellipticine to V79 cells is dependent on their expression of human CYP1A1, CYP1A2 or CYP3A4, these recombinant cells were treated with various concentrations (0.05–2 μM) of ellipticine for 48 hr. Ellipticine toxicity was dose-dependent with 2 μM ellipticine being lethal for all V79 cells (Fig. 4). Similarly, the IC_{50} values were not significantly different between cell lines The IC_{50} values calculated from the dose–log response

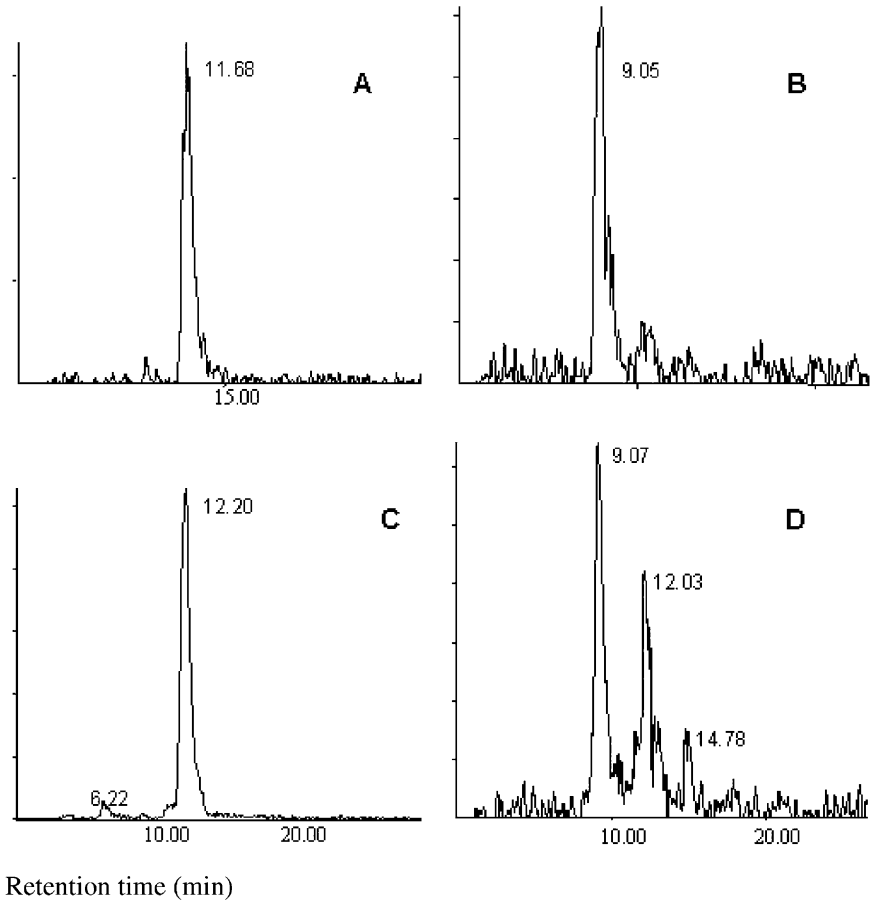

Fig. 3. Separation of ^{32}P-labelled nucleoside 3′,5′-bisphosphate adducts on a phenyl-modified reversed-phase column to compare adducts obtained *in vitro* (Fig. 1E) to those isolated from V79MZ-h3A4 cells (Fig. 1D). Adduct spots were excised and extracted from PEI-cellulose TLC plates, dissolved and injected into the HPLC system. (A) Spot 1 from Fig. 1E; (B) spot 2 from Fig. 1E; (C) spot 1 from Fig. 1D; (D) spot 2 from Fig. 1D. The abscissa shows the retention time (min), the ordinate radioactivity in arbitrary units.

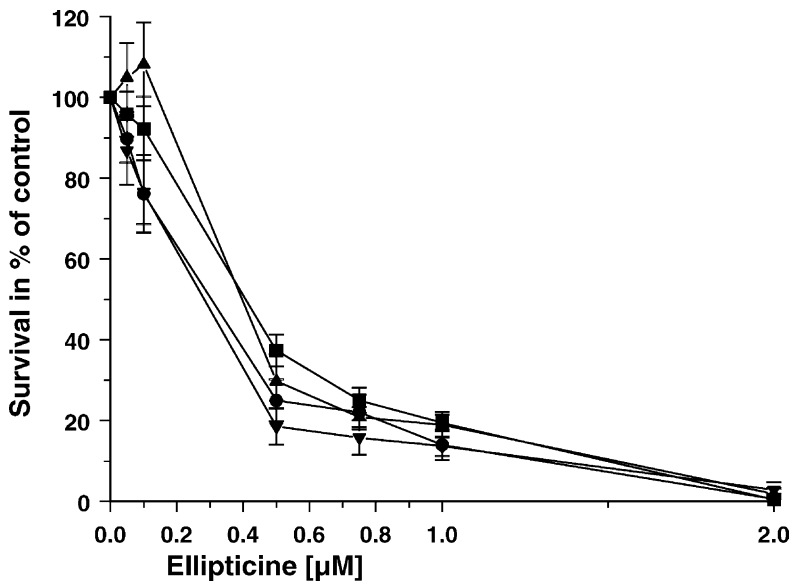

Fig. 4. Diagram showing cytotoxicity (viable cells as % of control) of ellipticine in the parental (V79MZ) and recombinant V79 cells used in the experiments after 48 hr exposure to different ellipticine concentrations, determined by the calcein assay. N = 8, values are means ± SD. (▲) V79 MZ, (●) V79MZ-h1A1, (▼) V79MZ-h1A2, (■) V79MZ-h3A4.

curves were 0.39 μM (V79MZ), 0.31 μM (V79MZ-h1A1), 0.25 μM (V79MZ-h1A2) and 0.37 (V79MZ-h3A4).

4. Discussion

In a previous study [1] we used two direct independent methods, namely ^{32}P-postlabelling and tritium-labelled ellipticine to show that ellipticine binds covalently to DNA *in vitro* after CYP-catalysed activation. In the present study we show with the ^{32}P-postlabelling technique that ellipticine binds covalently to DNA also in intact mammalian cells. The results clearly demonstrate that V79 cells expressing human CYP are appropriate model systems for the bioactivation of the anticancer drug ellipticine to DNA-binding species. The active derivatives binding to DNA generated in the cells seem to be the same as those formed *in vitro*, because the HPLC profiles of the isolated DNA adducts from cells and from *in vitro* incubations are the same. In contrast to the *in vitro* incubations 1–2 additional minor adducts were formed in cells expressing human CYP3A4, maybe by further oxidation of the intermediates generated by CYP, or by other oxidative enzymes constitutively expressed in V79 cells. We found that among the recombinant cells, those expressing human CYP3A4 had the highest DNA adduct levels (adduct spot 1), confirming our results with recombinant human CYP3A4 reconstituted with NADPH:CYP reductase or in microsomes (Figs. 1 and 3; [1]). Human CYP1A1 or CYP1A2 expressed in V79 cells also activated ellipticine to species forming adduct spot 1. This is again consistent with results from *in vitro* incubations of DNA with ellipticine and either of these CYP enzymes [1]. The identity of ellipticine-derived major DNA adducts formed in cell lines to those generated by purified enzymes *in vitro* was confirmed by two independent methods, by co-chromatography on TLC and by HPLC.

We can only speculate on the structure of ellipticine-DNA adducts shown in the present paper. One adduct (spot 2) is formed independently of enzyme activation (activated probably by auto-oxidation [1]), while the major adduct is formed upon catalysis by CYP. The chromatographic conditions used to resolve the adducts are suitable for hydrophobic, bulky adducts [17–19], therefore probably the whole ellipticine molecule is covalently linked to DNA. The ellipticine species, which reacts with DNA remains to be elucidated. However, it is probably not formed during ellipticine oxidation to 9-hydroxyellipticine, the main metabolite excreted in urine by humans, because CYP3A4, the predominant enzyme mediating the binding of ellipticine to DNA, forms very little 9-hydroxyellipticine (unpublished results).

In the mid 1980s the group of B. Meunier studied DNA binding of the antitumour drug N^2-methyl-9-hydroxyellipticinium, which was radioactively labelled, to DNA or RNA upon oxidation by, e.g. horseradish peroxidase [2,20,21]. The intermediate was found to be the 9-oxo derivative. In the case of CYP catalysed activation of ellipticine another mechanism seems to occur, because these authors found the non-hydroxylated ellipticinium derivative not to be oxidised and to bind only very weakly to DNA. This compound was also less toxic than the 9-hydroxy compound to L1210 cells.

In addition to the two known mechanisms of DNA damage (intercalation of ellipticine into DNA and generation of DNA strand breaks by inhibiting mammalian topoisomerase II), we have now found a third one, covalent binding of enzymatically-activated ellipticine. At the present time, it is not possible to demonstrate that antitumour, cytostatic and/or genotoxic activities of ellipticine are related to only one or several of these properties. The cytotoxicity of ellipticine to the V79 cells used in our studies was very strong and no significant differences between cells expressing CYP and the parental cells was observable. Thus, the cytotoxicity elicited by ellipticine towards V79 fibroblast cells in culture is independent of CYP expression and therefore does not seem to be related to covalent DNA adduct formation by this compound. Acute toxicity could be caused by the parent compound itself for instance by the inhibition of topoisomerase II activity, uncoupling mitochondrial oxidative phosphorylation thereby disrupting the cells' energy balance [9], or by CYP-independent metabolites.

Acknowledgments

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