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The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells

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ABSTRACT

Ellipticine is an antineoplastic agent, whose mode of action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of covalent DNA adducts mediated by cytochromes P450 and peroxidases. Here, the molecular mechanism of DNA-mediated ellipticine action in human neuroblastoma IMR-32, UKF-NB-3 and UKF-NB-4 cancer cell lines was investigated. Treatment of neuroblastoma cells with ellipticine resulted in apoptosis induction, which was verified by the appearance of DNA fragmentation, and in inhibition of cell growth. These effects were associated with formation of two covalent ellipticine-derived DNA adducts, identical to those formed by the cytochrome P450- and peroxidase-mediated ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine. The expression of these enzymes at mRNA and protein levels and their ability to generate ellipticine-DNA adducts in neuroblastoma cells were proven, using the real-time polymerase chain reaction, Western blotting analyses and by analyzing ellipticine-DNA adducts in incubations of this drug with neuroblastoma S9 fractions, enzyme cofactors and DNA. The levels of DNA adducts correlated with toxicity of ellipticine to IMR-32 and UKF-NB-4 cells, but not with that to UKF-NB-3 cells. In addition, hypoxic cell culture conditions resulted in a decrease in ellipticine toxicity to IMR-32 and UKF-NB-4 cells and this correlated with lower levels of DNA adducts. Both these cell lines accumulated in S phase, suggesting that ellipticine-DNA adducts interfere with DNA replication. The results demonstrate that among the multiple modes of ellipticine antitumor action, formation of covalent DNA adducts by ellipticine is the predominant mechanism of cytotoxicity to IMR-32 and UKF-NB-4 neuroblastoma cells.

1. Introduction

Neuroblastoma, a tumor of the peripheral sympathetic nervous system, is the most frequent solid extra cranial tumor in children and is a major cause of death from neoplasia in infancy [1]. These tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential [2]. Neuroblastoma may regress spontaneously in infants, mature to benign ganglioneuromas in older children, or grow relentlessly and be rapidly fatal [2]. Prognosis of high risk tumors is poor, because drug resistance arises in the majority of those patients, initially responding to chemotherapy, in spite of intensive therapy including megatherapy with subsequent hematopoietic progenitor cell transplantation, biotherapy and immunotherapy [2]. Little improvement in therapeutic options has been made in the last decade, requiring a need for the development of new therapies.

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1), an alkaloid isolated from *Apocyanacea* plants, and several of its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N^2-methyl-ellipticinium, 9-chloro-N^2-methyl-ellipticinium and 9-methoxy-N^2-methyl-ellipticinium) exhibit significant antitumor activities (for a summary see [3]). The main reasons for the interest

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Abbreviations: α-NF, α-naphthoflavone; COX, cyclooxygenase; CYP, cytochrome P450; Δc_T, difference of target minus reference cycle threshold; DMSO, dimethyl sulfoxide; IMDM, Iscove's modified Dulbecco's medium; HPLC, high-performance liquid chromatography; LPO, lactoperoxidase; MDR, multidrug resistance; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; PBS, phosphate buffered saline; PEI-cellulose, polyethylenimine-cellulose; PVDF, polyvinylidene difluoride; RAL, relative adduct labeling; RT, real-time; PCR, polymerase chain reaction; r.t., retention time; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography.

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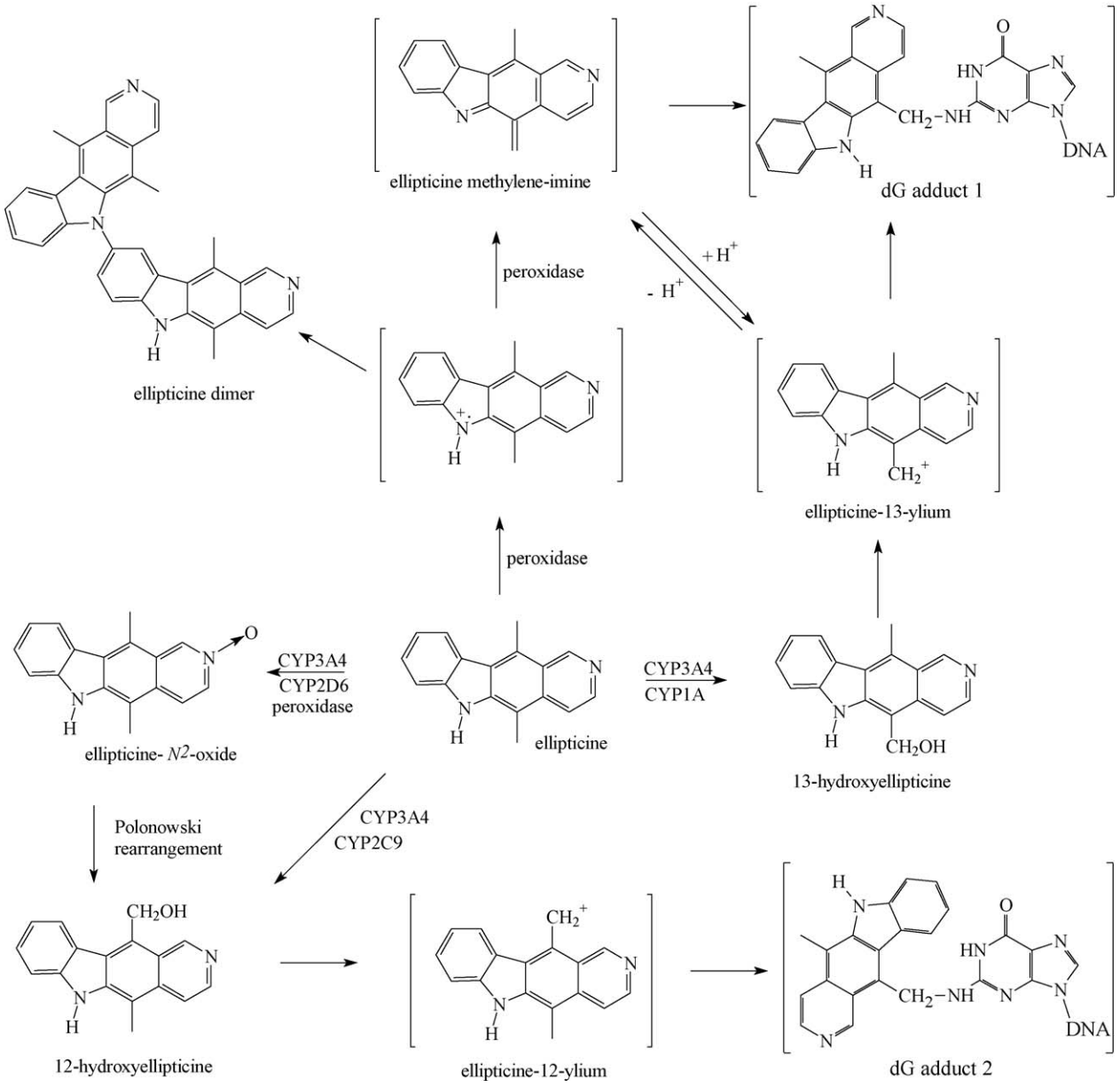

Fig. 1. Metabolism of ellipticine by peroxidases and human CYPs showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

in ellipticine and its derivatives for clinical purposes are their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity [4]. Nevertheless, ellipticine is a potent mutagen.

Ellipticine has been reported to arrest cell cycle progression by regulating the expression of cyclinB1 and Cdc2 as well as phosphorylation of Cdc2 [5,6], to induce apoptotic cell death by the generation of cytotoxic free radicals, the activation of Fas/Fas ligand system, the regulation of Bcl-2 family proteins [5–8], an increase of wild-type p53, the rescue of mutant p53 activity and the initiation of the mitochondrial apoptosis pathway [5,6,8,9]. Ellipticine also uncouples mitochondrial oxidative phosphorylation [10] and thereby disrupts the energy balance of cells. Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines [11,12], and this correlates with their cytotoxic activity. However, the precise molecular mechanism responsible for these effects has not yet been explained. Chemotherapy-induced cell cycle arrest was shown to result from DNA damages caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA [4,13] and (ii) inhibition of DNA topoisomerase II activity [4,14–16].

We have demonstrated that ellipticine also covalently binds to DNA *in vitro* and *in vivo* after being enzymatically activated with cytochromes P450 (CYP) or peroxidases [3,17–23], suggesting a third possible mechanism of action. Human and rat CYP1A, 1B1 and 3A are the predominant enzymes catalyzing oxidation of ellipticine *in vitro* either to metabolites that are excreted (7-hydroxy- and 9-hydroxyellipticine) or that form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) [3,17–20]. Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine

lactoperoxidase (LPO), human myeloperoxidase (MPO) and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts (Fig. 1) [21,23]. CYP- and/or peroxidase-mediated ellipticine-DNA adducts were detected also in rats and mice *in vivo* [18,22,24]. The same DNA adducts were also detected in cells in culture expressing enzymes activating ellipticine (CYP1A1, COX-1 and MPO), such as human breast adenocarcinoma MCF-7 cells [25], leukemia HL-60 and CCRF-CEM cells [26] and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 [27]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its activation by CYPs and peroxidases in target tissues.

The aims of this study were to test the cytotoxicity of ellipticine on a panel of human neuroblastoma cell lines [28], including lines resistant to several anticancer drugs [29] and to examine whether DNA adducts are formed in these human cancer cells. The ^{32}P-postlabeling method was used to determine DNA adduct formation by ellipticine and cytotoxicity of ellipticine was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay [30]. Furthermore, other biological parameters including cell-cycle apoptosis and expression of enzymes activating ellipticine were investigated in this study. In addition, we wanted to establish the molecular mechanisms of anticancer action of ellipticine in neuroblastoma cells and to evaluate whether resistance to ellipticine might be induced upon long-term exposure of these cells to this drug.

2. Materials and methods

2.1. Chemicals

Ellipticine, calf thymus DNA, α-naphthoflavone (α-NF), ketoconazole, MTT, N,N-dimethylformamide, agarose, methanol, ethanol, Tris, RNase, proteinase K, propidium iodide, sodium dodecyl sulphate (SDS), polyethylene glycol 6000 and NADPH were from Sigma (St. Louis, MO, USA), dimethyl sulfoxide (DMSO) from Amresco Inc. (Solon, OH, USA), EDTA, Na_2HPO_4, NaH_2PO_4, H_3PO_4, acrylamide, 1% Triton X-100 from Fluka Chemie AG (Buchs, Switzerland), hydrogen peroxide from Merck (Darmstadt, Germany), doxorubicin from Ebewe Pharma (Unterach, Austria), phenol–chloroform from Roth (Karlsruhe, Germany), serum albumin, ammonium hydroxide, isopropanol from PLIVA-Lachema (Brno, Czech Republic). 12-Hydroxy- and 13-hydroxyellipticine were isolated from multiple HPLC runs of ethyl acetate extracts of incubations containing ellipticine and human and/or rat hepatic microsomes as described [19]. All these and other chemicals used in the experiments were of analytical purity or better. Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described [3].

2.2. Cell cultures

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high risk neuroblastoma, were a gift of Prof. J. Cinatl Jr. (J. W. Goethe University, Frankfurt, Germany). The cell line UKF-NB-4 was established from chemoresistant recurrency. IMR-32, high risk neuroblastoma derived cell line, was of commercial source (LGC Promochem, Wesel, Germany). All three cell lines used were derived from high risk neuroblastoma with MYCN amplification, del1p and aneuploidy. The vincristine, doxorubicin, cisplatin and ellipticine resistant cell sublines designated UKF-NB-3 (VCR), UKF-NB-3 (DOXO), UKF-NB-4 (DOXO), UKF-NB-4 (cisplatin) and UKF-NB-4 (Elli) were established by incubation of parental cells with increasing concentrations of the respective drug by a procedure described [31]. Cells were grown at 37 °C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 μg/ml streptomycine (PAA Laboratories, Pasching, Austria). For hypoxia experiments, cells were maintained in modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) flushed with 1% O_2, 5% CO_2 and balance N_2 for 4 min at 37 °C (hereafter referred to as hypoxia).

2.3. MTT assay

The cytotoxicity of ellipticine and doxorubicin to cells in exponential growth was determined in a 96-well plate. For a dose–response curve, cells in exponential growth were seeded in 100 μl of medium with 10^4 cells per well. Solution of ellipticine and doxorubicin in DMSO (5 μl) in final concentrations of 0.02–50 μM was added. Control cells and medium controls without cells received 5 μl of DMSO without drug. Tumor cell viability was evaluated by MTT test as previously described [30]. Briefly, after incubation (2–4 days) at 37 °C in 5% CO_2 saturated atmosphere the MTT solution (2 mg/ml) was added, the plates were incubated for 4 h and cells lysed in PBS containing 20% of SDS and 50% N,N-dimethylformamide, pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular Devices, Sunnyvale, CA, USA). The mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviations. The IC_{50} values were calculated from the linear regression of the dose–log response curves by SOFTmaxPro.

2.4. Cell cycle analysis

To determine cell cycle distribution analysis, 5×10^5 cells were plated in 60 mm dishes and treated with ellipticine (0, 1 and 10 μM) for 48 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, resuspended in 1 ml of PBS containing 1 μg/ml RNase and 50 mg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by flow cytometry on a FACSCalibur cytometer (BD, San Jose, CA, USA). The data were analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA).

2.5. Apoptosis assay

Cells (5×10^6) were treated with vehicle alone (5 μl DMSO) and increasing concentrations of ellipticine for 48 h, and then collected by centrifugation. Pellets were lysed by DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM EDTA, and 1% Triton X-100) and centrifuged. The supernatant obtained was incubated overnight with proteinase K (0.1 mg/ml) and with RNase (0.2 mg/ml) for 2 h at 37 °C. After extraction with phenol–chloroform (1:1), the DNA was separated in 2% agarose gel (Amresco Inc., Solon, OH, USA) and visualized by UV after staining with ethidium bromide (Top-Bio s.r.o., Prague, Czech Republic).

2.6. Preparation of S9 fractions and assays

S9 fractions were isolated from IMR-32, UKF-NB-3 and UKF-NB-4 cancer cell homogenates by fraction centrifugation as described [32] and the 10,000×*g* for 15 min supernatants were used for incubations.

2.7. Incubations of ellipticine with neuroblastoma S9 fractions

Incubation mixtures used to generate DNA adducts by ellipticine consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, S9 fractions (0.5 mg protein) from IMR-32, UKF-NB-3 and UKF-NB-4 cells, 0.1 mM ellipticine (dissolved in 7.5 μl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 μl. Incubations were also carried out in the presence of a peroxidase cofactor, hydrogen peroxide (0.1 mM) [33,34]. Incubations were carried out at 37 °C for 30 min; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation [3]. Control incubations were carried out (i) without S9 fraction, (ii) without NADPH or hydrogen peroxide, (iii) without DNA and (iv) without ellipticine. After the incubation, DNA was isolated by a standard phenol–chloroform extraction method.

2.8. Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine to form DNA adducts in the presence of S9 subcellular fraction of neuroblastoma cells: α-NF, which inhibits CYP1A1 and 1A2 [34–36], and activates oxidation of some substrates by CYP3A4 [36], and ketoconazole, an inhibitor of CYP3A4 [36]. Inhibitors were dissolved in 7.5 μl of methanol, to yield final concentrations of 0.1 mM in the incubation mixtures. Mixtures were then incubated at 37 °C for 10 min with NADPH prior to adding ellipticine, and then incubated for a further 30 min at 37 °C. After the incubation, DNA was isolated as described above, and analyzed for ellipticine-DNA adduct formation.

2.9. Isolation of CYP enzymes and preparation of antibodies against these enzymes

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA [37]. Human recombinant CYP3A4 was a gift of P. Anzenbacher (Palacky University, Olomouc, Czech Republic).

Leghorn chickens were immunized subcutaneously three times a week with CYP antigens (rat recombinant CYP1A1 and human recombinant CYP3A4 emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters). Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 [37,38].

Commercial antibodies, rabbit anti-human CYP1B1 (AbCam, Cambridge, UK) and mouse monoclonal anti-human COX-1 and -2 (Exalpha, Shirley, MA, USA), were used in the experiments.

2.10. Estimation of contents of CYPs, COX-1 and -2 in neuroblastoma cells

Immunoquantitation of CYPs (CYP1A1, 1B1 and 3A4) and COX-1 and -2 in homogenates of neuroblastoma cells was done by Western blot. Protein concentrations in homogenates were assessed using the bicinchoninic acid protein assay (Pierce Rockford, IL, USA) with serum albumin as a standard [18]. Samples containing 10–45 μg proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels [37,38]. After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) (Millipore, Billerica, MA, USA) and/or nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Cellular CYP and COX-1 and -2 proteins were probed with the anti-CYP and anti-COX-1 and -2 rabbit and mouse polyclonal antibodies, respectively, as reported elsewhere [26,37,38]. The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and 1A2 in rat and human liver microsomes [37–39]. Human recombinant CYP1A1, 1B1 and 3A4 (in Supersomes™, Gentest Corp., Woburn, MA, USA), ovine COX-1 and human recombinant COX-2 (Gentest Corp., Woburn, MA, USA) were used as positive controls to identify the bands of these enzymes in cellular homogenates. The antigen–antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as dye [37,38] and/or with chemiluminescence reagents (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA). Membranes were scanned with a computerized image-analyzing system (Imstar). The detection limit was 0.005 pmol CYP1A per lane (see also literature [37,38]) and 0.01 pmol for the other enzymes.

2.11. Estimation of MPO content in neuroblastoma cells

MPO was analyzed by flow cytometry using anti-human MPO-FITC antibody (Immunotech, Marseille, France). Cultivation was performed in 12-well plates, three samples from every well were prepared and two wells measured. Cells were permeabilized with Fix and Perm kit (Caltag Laboratories, Burlingame, CA, USA) according to the producer's recommendation. The fluorescence intensity of at least 10,000 cells was measured by FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with 488 nm laser and list mode data were analyzed with the CellQuest software [40]. Expression was evaluated as mean intensity of fluorescence. The fluorescence measurements were calibrated for each run by FITC-conjugated bead standards (DAKO Glostrup, Denmark).

2.12. CYP1A1, 1B1, 3A4, COX-1 and -2 mRNA content in neuroblastoma cells

Total RNA was isolated from neuroblastoma cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis and RNA quantity was assessed by UV–vis spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA, USA). RNA samples (1 μg) were reversely transcribed using random hexamer primers utilizing High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The prepared cDNA was used for real-time (RT) polymerase chain reaction (PCR) under the following cycling conditions: incubation at 50 °C for 2 min and initial denaturation at 95 °C for 10 min, then 50 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The PCR reaction mixtures (20 μl) contained 2 μl cDNA, 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and 2 μl of primer and probe mixture (commercially available unlabeled PCR primers and FAM™ dye-labeled probe for human *CYP1A1*, *1B1*, *3A4*, *COX-1* and *-2* as target genes and *β-actin* as reference internal standard gene) produced by Generi Biotech (Hradec Kralove, Czech Republic). Each sample was analyzed in three parallel aliquots. Negative controls had the same composition as samples but ultrapure water was used instead of cDNA. Samples were analyzed by ABI 7300 cycler (Applied Biosystems, Foster City, CA, USA) and data were evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then Δc_T was evaluated according to the following equations: $\Delta c_T = c_T$ (target) $- c_T$ (internal standard). Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. The changes in mRNA expression of studied target genes in UKF-NB-4 (Elli) cells compared to the parental line was calculated with $2^{-(\Delta\Delta c_T)}$.

2.13. Treatment of neuroblastoma cells with ellipticine for DNA adduct analyses

Neuroblastoma cell lines were seeded 24 h prior to treatment at a density of 1×10^5 cells/ml in two 75 cm^3 culture flasks in a total volume of 20 ml of IMDM. Ellipticine was dissolved in 20 μl of DMSO, the final concentration was 0, 0.1, 1 or 10 μM. After 48 h the cells were harvested after trypsinizing by centrifugation at 2000×g for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at −20 °C until DNA isolation. DNA was isolated and labeled as described in the next section.

2.14. DNA isolation and ^{32}P-postlabeling of DNA adducts

DNA from cells was isolated by the phenol–chloroform extraction as described [25,27]. ^{32}P-postlabeling analyses were performed using nuclease P1 enrichment as described previously [3,19,21,27]. From experiments performed earlier, calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine [19,21], with ellipticine and human recombinant COX-2 [21], and liver DNA of rats treated with ellipticine [17] were labeled with ^{32}P to compare adduct spot patterns.

2.15. HPLC analysis of ^{32}P-labeled DNA adducts

HPLC analysis was performed essentially as described previously [18]. Individual spots detected by ^{32}P-postlabeling were excised from the thin layer and extracted [18]. Cut-outs were extracted with two 800 μl portions of 6 M ammonium hydroxide/isopropanol (1:1) for 40 min. The eluent was evaporated in a Speed-Vac centrifuge. The dried extracts were dissolved in 100 μl of methanol/phosphate buffer (pH 3.5) 1:1 (v/v). Aliquots (50 μl) were analyzed on a phenyl-modified reversed-phase column (250 mm × 4.6 mm, 5 μm Zorbax Phenyl; Säulentechnik Knauer, Berlin, Germany) 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 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).

2.16. Statistical analyses

Statistical associations between IC_{50} values for ellipticine in human neuroblastoma cells and levels of total ellipticine-derived DNA adducts formed in the same cells were determined by the linear regression using Statistical Analysis System software version 6.12. All *P*-values are two-tailed and considered significant at the 0.05 level.

3. Results

3.1. Cytotoxicity of ellipticine to human neuroblastoma cells

To determine the cytotoxicity of ellipticine to human neuroblastoma cells, these cells were treated with increasing concentrations of ellipticine. We first determined the effect of ellipticine on growth of human neuroblastoma cell lines (IMR-32, UKF-NB-3 and UKF-NB-4) cultured for 96 h in the presence of ellipticine, using MTT assay. As shown in Fig. 2, all three neuroblastoma cell lines were sensitive to ellipticine. The cytotoxic effects of ellipticine were also detected to the derived daughter neuroblastoma lines that were resistant to vincristine, doxorubicin or cisplatin (Table 1).

Cytotoxicity of ellipticine was compared with that of doxorubicin (Table 1), one of the drugs currently used for neuroblastoma

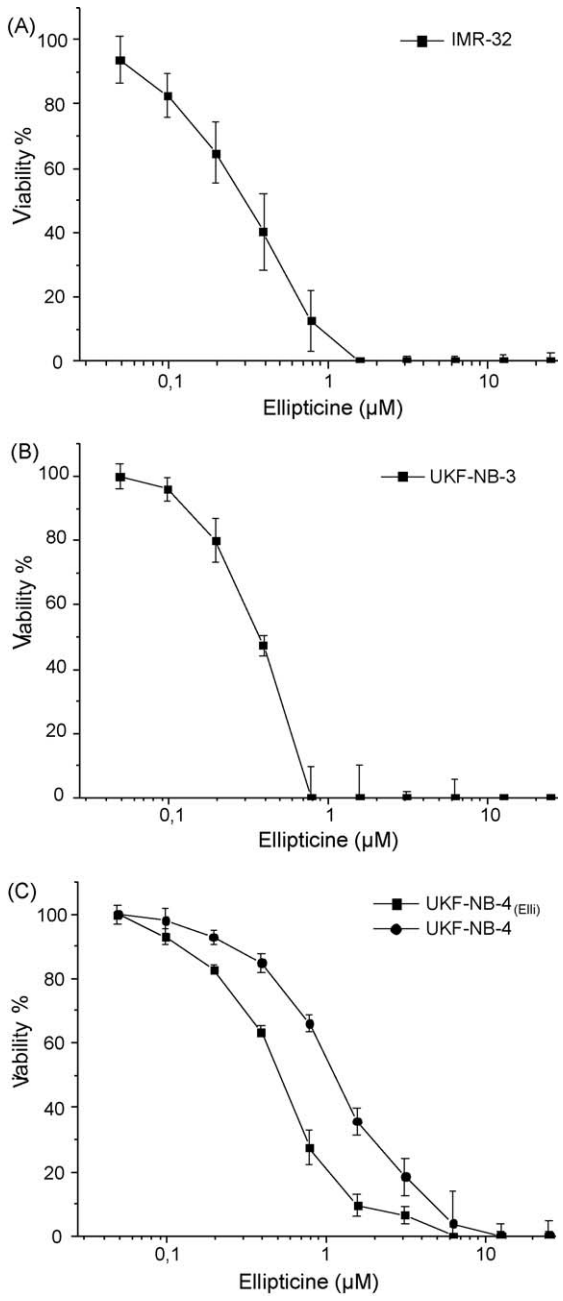

Fig. 2. Cytotoxicity (viable cells as percent of control) of ellipticine to IMR-32 (A), UKF-NB-3 (B), UKF-NB-4 and UKF-NB-4 cells resistant to ellipticine (UKF-NB-4^{Elli}) (C) after 96 h exposure to the compound, determined by the MTT assay. Values are means and standard deviations of 8 determinations.

treatment [2]. Ellipticine and doxorubicin inhibited the growth of neuroblastoma cell lines in a dose-dependent manner. The IC_{50} values for ellipticine and doxorubicin calculated from the dose-log response curves are shown in Table 1. The toxicity of ellipticine to UKF-NB-3 and UKF-NB-4 cells was similar to that of doxorubicin to these cells, while IMR-32 cells were more than 20-times more sensitive to doxorubicin than to ellipticine (see IC_{50} values shown in Table 1).

Cytotoxicity elicited by ellipticine and/or doxorubicin in the parental cell lines was compared to that in their variants resistant to doxorubicin. Neuroblastoma cells resistant to doxorubicin were only slightly resistant to ellipticine (Table 1), even though cross-resistance is expected [31]. The IC_{50} values for ellipticine in IMR-32 (DOXO), UKF-NB-3 (DOXO) and UKF-NB-4 (DOXO) were only 2-,

Table 1
Cytotoxicity of ellipticine and doxorubicin to neuroblastoma cell lines.

Cells	IC_{50}	
	For ellipticine (μM)	For doxorubicin (μM)
IMR-32	0.27 ± 0.02	0.01 ± 0.01
IMR-32 (hypoxic conditions)	0.43 ± 0.02***	Not measured
IMR-32 (DOXO)	0.53 ± 0.03***	0.10 ± 0.03***
UKF-NB-3	0.44 ± 0.03	0.42 ± 0.04
UKF-NB-3 (hypoxic conditions)	0.44 ± 0.03	0.47 ± 0.04
UKF-NB-3 (VCR)	0.64 ± 0.05***	0.83 ± 0.08***
UKF-NB-3 (DOXO)	0.78 ± 0.06***	1.75 ± 0.17***
UKF-NB-3 (cisplatin)	0.46 ± 0.02	0.69 ± 0.05***
UKF-NB-4	0.44 ± 0.03	0.70 ± 0.02
UKF-NB-4 (hypoxic conditions)	0.77 ± 0.04***	0.60 ± 0.05**
UKF-NB-4 (Elli)	1.17 ± 0.07***	0.70 ± 0.05
UKF-NB-4 (Elli) (hypoxic conditions)	1.57 ± 0.08***ΔΔΔ	1.51 ± 0.09***ΔΔΔ
UKF-NB-4 (DOXO)	1.14 ± 0.07***	3.80 ± 0.12***
UKF-NB-4 (cisplatin)	0.62 ± 0.05***	0.22 ± 0.02***

IC_{50} values were calculated from the linear regression of the dose–log response curves. Values are mean ± S.D. of at least three experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from individual parent cell lines cultivated under the standard conditions: $^{**}P < 0.01$, $^{***}P < 0.001$. Values significantly different from the UKF-NB-4 (Elli) cell line cultivated under the standard conditions: $^{\Delta\Delta\Delta}P < 0.001$.

1.8- and 2.6-fold higher than those in the parental cell lines, respectively, whereas the IC_{50} values for doxorubicin were up to 10-fold higher in the doxorubicin-resistant cell lines. Likewise, neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines resistant to vicristine and cisplatin showed practically no cross-resistance to ellipticine; the IC_{50} values for ellipticine in UKF-NB-3 (VCR) and UKF-NB-4 (cisplatin) were only 1.4-fold higher than those in the parental cell lines (Table 1). The IC_{50} values for ellipticine in UKF-NB-3 and UKF-NB-3 (cisplatin) were even identical (Table 1).

3.2. Ellipticine-induced cell cycle arrest and apoptosis in human neuroblastoma cells

In order to examine the mechanism responsible for ellipticine-mediated cell growth inhibition, we first evaluated cell cycle distribution using flow cytometric analysis. Compared with the vehicle-treated control, treatment cells with 10 μM ellipticine resulted in an appreciable arrest of IMR-32 and UKF-NB-4 cells in S phase of cell cycle after 48 h of treatment with a concomitant decrease in G0/G1 phase (Fig. 3). On the contrary, different effects of ellipticine on the cell cycle of UKF-NB-3 cells were detected; an arrest in G0/G1 and G2/M phases of cell cycle with a concomitant pronounced decrease in S phase was found in this neuroblastoma line.

We next assessed the effect of ellipticine on the induction of apoptosis in neuroblastoma cells by DNA fragmentation assay. The results showed that ellipticine treatment results in the formation of DNA fragments in neuroblastoma cells, as assessed by agarose gel electrophoresis at 48 h (see Fig. 4 for UKF-NB-4 cells).

3.3. Determination of DNA adduct formation by ellipticine in neuroblastoma cells

The three parental neuroblastoma cell lines shown to be sensitive to ellipticine (Table 1) were treated with 0.1, 1, and 10 μM ellipticine for 48 h. Using the nuclease P1 version of ^{32}P-postlabeling assay, which was found to be suitable to detect and quantify DNA adducts formed by ellipticine [3,17–19,21,22], ellipticine-derived adducts were detected in the DNA of these cells (Fig. 5A–C). Two major ellipticine-DNA adducts (spots 1 and 2 in Fig. 5A–C) were formed in all cells (Table 2). No adducts were detected in DNA of control cells treated with solvent only.

Chromatographic properties of the two major adduct spots on PEI-cellulose TLC plates (spots 1 and 2) were similar to those of ellipticine-derived DNA adducts found previously after *in vitro* incubation of calf thymus DNA with ellipticine and isolated CYPs [3,18], or peroxidases [21] or after treatment of cells in culture with this anticancer drug [25–27] or *in vivo* (Fig. 5D), in several organs of rats [17,22] and mice [24] exposed to this agent. Both these adducts were found to be generated from 13-hydroxy- and 12-hydroxyellipticine [19,21,41] (Fig. 5E and F) as confirmed by cochromatographic analysis using TLC and HPLC (data not shown). Both adducts were identified as deoxyguanosine adducts in DNA [18]. Besides these adducts, additional two minor adducts (spots 6 and 7 in Fig. 5C and D) were detected in DNA of UKF-NB-4 cells treated with 10 μM ellipticine (Table 2). Both these minor adducts are known to be generated *in vitro* mainly by peroxidase-catalyzed oxidation [21,23]. The low levels of these adducts prevented HPLC co-chromatographic analysis or their further characterization.

Ellipticine-DNA adduct levels were dose dependent in all cells with an overproportional increase between 1 and 10 μM ellipticine. IMR-32 cells resistant to doxorubicin formed lower levels of ellipticine-DNA adducts as did UKF-NB-4 cells resistant to this drug than the respective parental cells. All resistant daughter cells of these lines except those to cisplatin had lower ellipticine-DNA adduct levels. The highest levels over all were formed in UKF-NB-3 resistant to cisplatin (Table 2).

3.4. Ellipticine-resistant neuroblastoma cells

To induce resistance to ellipticine, UKF-NB-4 cells were incubated for 36 months with increasing concentrations of ellipticine (1–2.5 μM). Morphological changes were seen in these UKF-NB-4 cells (Fig. 6), which did not change during five passages in medium without ellipticine. These cells were indeed resistant, since IC_{50} values of ellipticine increased 2.7-fold (Fig. 2 and Table 1). In addition, the growth rate of the ellipticine-resistant UKF-NB-4 cell line was slower than that of the sensitive line (see doubling times shown in Table 3). The development of resistance of the UKF-NB-4 cells resulted in 3.5-fold lower levels of ellipticine-DNA adducts after treatment of these cells with ellipticine (Table 2). These effects were associated also with changes in ellipticine-induced cell cycle arrest. While 1 and 10 μM ellipticine induced an arrest in the S phase of the cell cycle in the parent neuroblastoma cell line, no significant arrest in this phase was detectable in the ellipticine-resistant UKF-NB-4 cell line at 1 μM ellipticine; only a minor arrest in this phase of the cell cycle ($P < 0.05$) was induced by 10 μM ellipticine. In addition, a low but significant arrest in G2/M phase of cell cycle with a concomitant decrease in G0/G1 phase was caused by ellipticine in the resistant line ($P < 0.001$ at 10 μM ellipticine) (Fig. 3).

3.5. Expression of biotransforming enzymes in human neuroblastoma cells

Using Western blot analysis with polyclonal antibodies raised against CYP1A1, 1B1 and 3A4 or with monoclonal antibodies raised against COX-1 or -2, the protein expression levels of these enzymes were analyzed in the parental neuroblastoma lines and in the cells resistant to ellipticine. Under the experimental conditions used, expression of CYP1A, 1B1 and 3A4 (Fig. 7) was found in all cells, while no expression of COX-1 and -2 was detected. MPO expression was analyzed by flow cytometry, but was not detected in any of the neuroblastoma cells analyzed (data not shown).

Besides protein expression of the enzymes, mRNA expression in neuroblastoma cells was also investigated. Total RNA was isolated

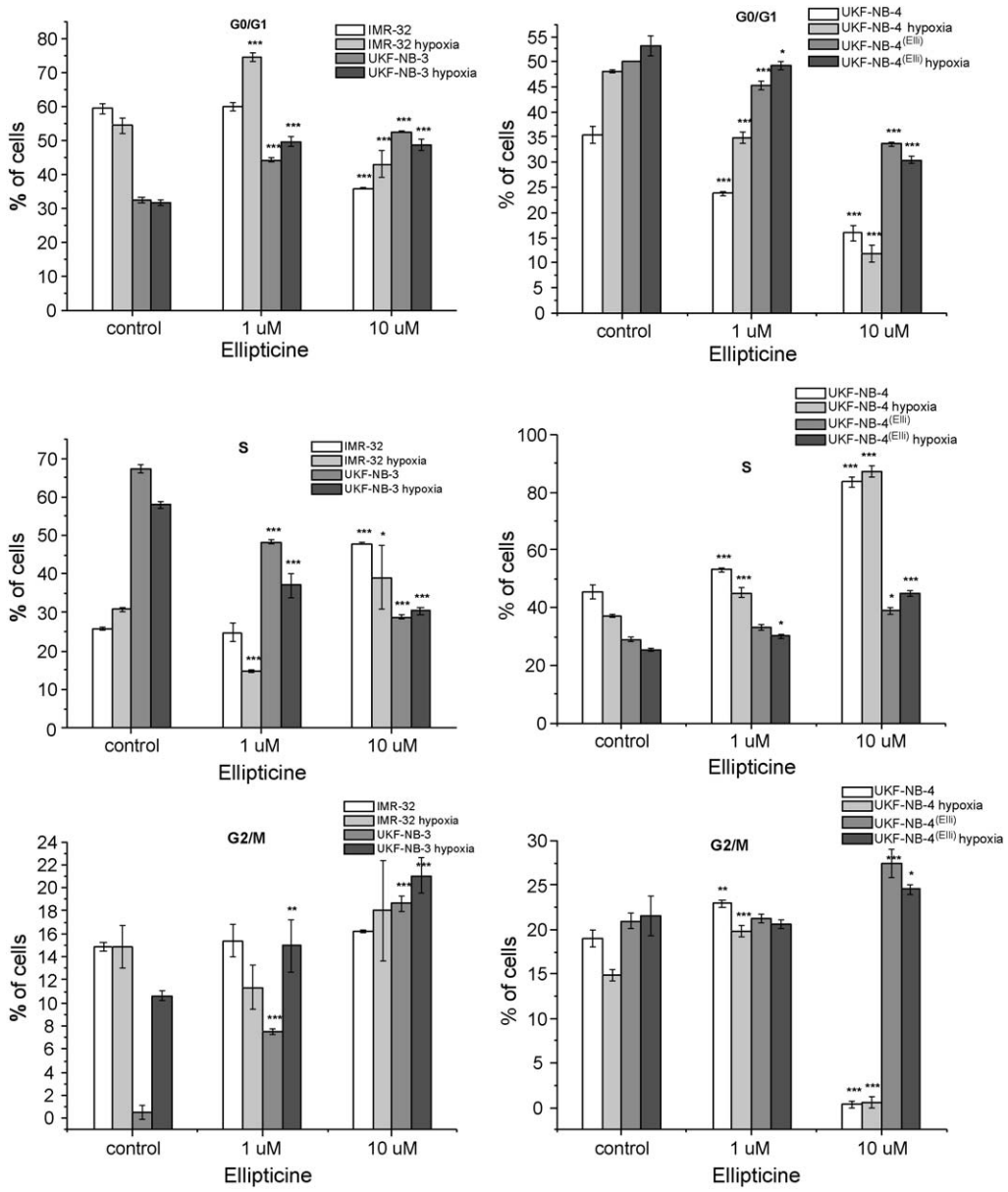

Fig. 3. Effect of ellipticine on cell cycle distribution in neuroblastoma cells. Cell were treated with vehicle or ellipticine for 48 h, and cell cycle distribution was assessed by flow cytometry. $N = 3$, values are means ± S.D.

from frozen cells and the relative amounts of *CYP1A1*, *1B1*, *3A4*, *COX-1* and *-2* mRNAs were measured by real-time PCR. Even though expression of mRNAs of these enzymes was detectable in all neuroblastoma cell lines, levels of *COX-2*, *CYP3A4* and *1A1* mRNAs were extremely low in the three original lines (Table 4). In UKF-NB-4 cell resistant to ellipticine, 0.5- and 0.3-fold lower levels of *CYP3A4* and *1B1* mRNAs, respectively, were found than in the parental line (Table 4). On the contrary, higher levels of *CYP1A1* (10.3-fold) and *COX-2* mRNAs (300.6-fold) were detected in the ellipticine-resistant cells line (Table 4). The changes in mRNA expression of studied target genes in UKF-NB-4 (Elli) cells compared to the parental line were calculated with $2^{-(\Delta\Delta cT)}$. However, if cells of both lines were exposed to 10 μM ellipticine, no difference in expression levels of these mRNAs was found between the ellipticine-resistant and sensitive UKF-NB-4 cell lines; levels of *CYP1A1*, *1B1*, *3A4*, *COX-1* and *-2* mRNAs were essentially the same and similar to those found in unexposed cells (data not shown).

3.6. S9 fractions of neuroblastoma cells are capable of activating ellipticine

Because CYPs and peroxidases, which activate ellipticine [3,17,19,22], were found to be expressed in neuroblastoma cells (see above), we evaluated whether S9 fractions isolated from the parental IMR-32, UKF-NB-3 and UKF-NB-4 cells activate ellipticine to species forming DNA adducts.

The DNA adduct pattern generated by ellipticine in this system was determined by ^{32}P-postlabeling, and again consisted of two adducts, which were identical to adduct spots 1 and 2 formed in intact neuroblastoma cells (Fig. 5A–C and Table 5). Chromatographic analysis of spots 1 and 2 on HPLC confirmed that these adducts are derived from 13-hydroxy- and 12-hydroxyellipticine, respectively, by their coelution with prepared reference compounds (data not shown). Control incubations without S9 subcellular fractions were free of adduct spot 1, but adduct spot 2 was always detected (data not shown). This finding is consistent

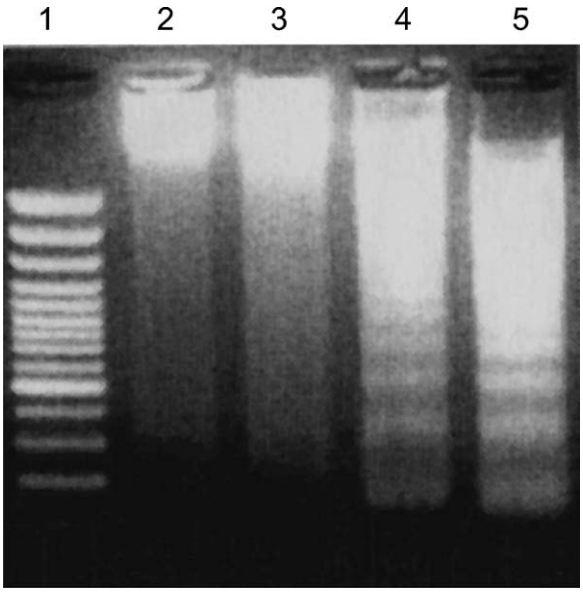

Fig. 4. The induction of apoptosis in ellipticine-treated neuroblastoma UKF-NB-4 cells. The DNA fragmentation was assessed by gel electrophoresis. Cells were treated with vehicle and ellipticine for 48 h, and then the fragmentation of DNA was assessed by agarose gel electrophoresis. (Lane 1) marker, (lane 2) UKF-NB-4 cells treated with vehicle alone (DMSO), (lane 3) 0.1 μM ellipticine, (lane 4) 1 μM ellipticine, and (lane 5) 10 μM ellipticine.

with our previous results showing that this adduct is formed also non-enzymatically [3,18,19,21].

To determine the extent of CYP and peroxidase contribution to ellipticine activation, we added cofactors and selective inhibitors of these enzymes to incubations containing S9 fractions isolated from neuroblastoma cells. In the presence of NADPH, the cofactor of CYP, S9 samples of all cells were capable of activating ellipticine to form DNA adducts (Table 5). The S9 fraction from IMR-32 cells was most active.

α-NF, an inhibitor of CYP1A and an activator of CYP3A4 [36], slightly decreased the levels of ellipticine-DNA adducts formed by the S9 fraction of UKF-NB-3 cells (Table 5), but had no effect in incubation with S9 fraction from UKF-NB-4 cells. In contrast, α-NF added to the S9 fraction of IMR-32 cells led to 3-fold higher levels of ellipticine-DNA adducts, predominantly of adduct 1, suggesting the importance of CYP3A4 in ellipticine activation in these cells (Table 5). This suggestion was confirmed by the effect of ketoconazole, a selective inhibitor of CYP3A [36,42], which significantly decreased the levels of ellipticine-DNA adducts formed by S9 fractions from all three cell lines. Addition of hydrogen peroxide, a cofactor for peroxidases [21,33,34,43], increased ellipticine-DNA adduct levels in S9 fractions of all neuroblastoma cells, most effectively in UKF-NB-4 cells, where 10-fold higher levels than without cofactor were determined (Table 5).

These results demonstrate that CYPs and peroxidases are active to different degree in neuroblastoma cells from different patients, and capable of catalyzing the formation of DNA adducts 1 and 2 from ellipticine.

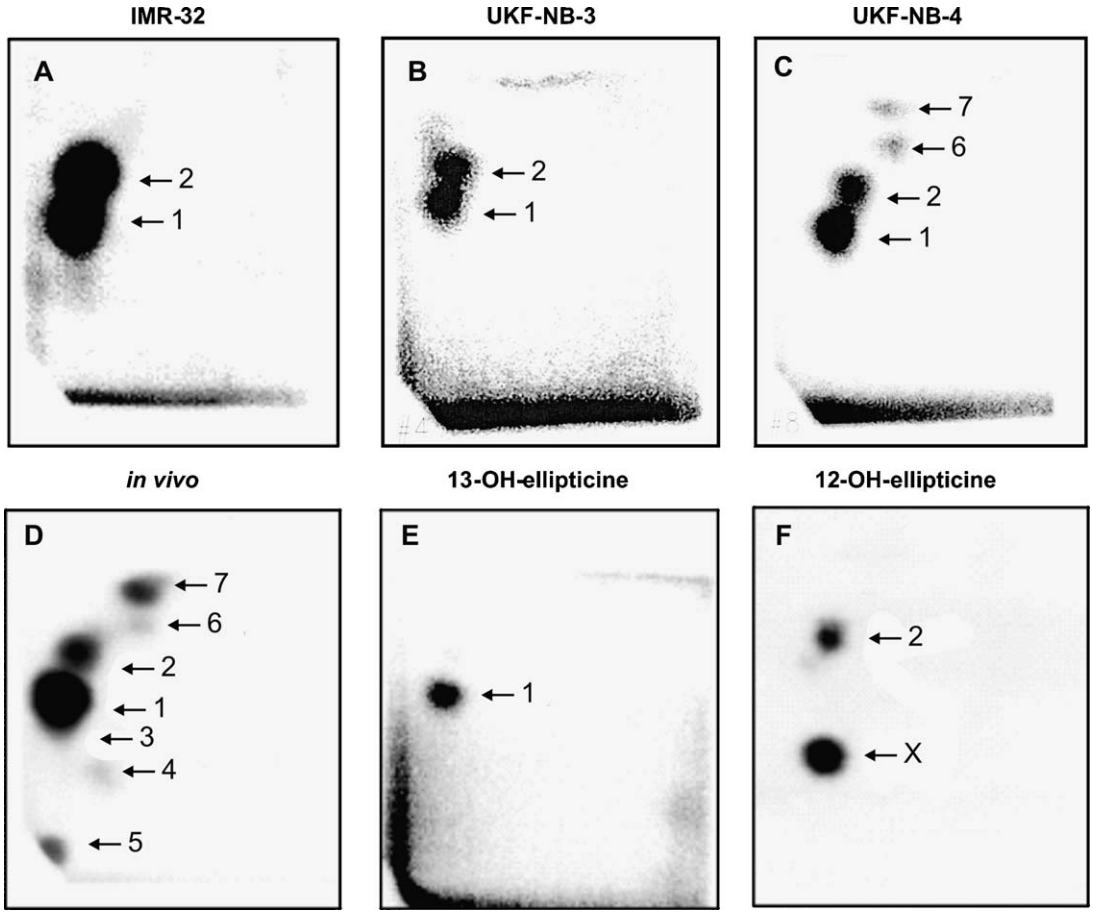

Fig. 5. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from neuroblastoma IMR-32 (A), UKF-NB-3 (B) and UKF-NB-4 cells exposed to 10 μM ellipticine (C) for 48 h, of liver DNA of rats treated with 40 mg ellipticine per kilogram body weight (D), from calf thymus DNA reacted with 13-hydroxyellipticine (E) and 12-hydroxyelipticine (F). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. (A, B, D) Scans of the plates for 6.5 min from the imager; (C, E, F) autoradiographs of films exposed for 1 h at −80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

Table 2
DNA adduct formation by ellipticine in human neuroblastoma cell lines.

Cells	Levels of DNA adducts (RAL × 10^{-7})[a]				
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total
IMR-32					
+0.1 μM ellipticine	0.10 ± 0.01	0.13 ± 0.01	n.d.	n.d.	0.23 ± 0.02
+1.0 μM ellipticine	0.26 ± 0.02	0.31 ± 0.03	n.d.	n.d.	0.57 ± 0.05
+1.0 μM ellipticine hypoxic conditions	0.22 ± 0.03*	0.23 ± 0.02***	n.d.	n.d.	0.45 ± 0.04**
+10 μM ellipticine	13.15 ± 1.30	13.13 ± 1.30	n.d.	n.d.	26.28 ± 2.60
+10 μM ellipticine hypoxic conditions	8.88 ± 1.01***	7.22 ± 0.82***	n.d.	n.d.	16.10 ± 1.63***
IMR-32 (DOXO)					
+0.1 μM ellipticine	0.02 ± 0.01	0.12 ± 0.01	n.d.	n.d.	0.14 ± 0.01
+1.0 μM ellipticine	0.17 ± 0.02	0.30 ± 0.03	n.d.	n.d.	0.47 ± 0.05
+10 μM ellipticine	2.88 ± 0.29	7.14 ± 0.70	n.d.	n.d.	10.02 ± 1.00
UKF-NB-3					
+1.0 μM ellipticine	0.12 ± 0.01	0.23 ± 0.02	n.d.	n.d.	0.35± 0.04
+1.0 μM ellipticine hypoxic conditions	0.18 ± 0.02**	0.15 ± 0.02***	n.d.	n.d.	0.33 ± 0.03
+10 μM ellipticine	3.26 ± 0.32	2.64 ± 0.40	n.d.	n.d.	5.90 ± 0.68
+10 μM ellipticine hypoxic conditions	1.63 ± 0.21***	1.65 ± 0.19***	n.d.	n.d.	3.28 ± 0.42***
UKF-NB-3 (VCR)					
+0.1 μM ellipticine	0.04 ± 0.01	0.04 ± 0.01	n.d.	n.d.	0.08 ± 0.02
+1.0 μM ellipticine	0.27 ± 0.03	0.62 ± 0.06	n.d.	n.d.	0.89 ± 0.09
+10 μM ellipticine	6.49 ± 0.65	10.14 ± 1.00	n.d.	n.d.	16.63 ± 1.70
UKF-NB-3 (DOXO)					
+0.1 μM ellipticine	0.13 ± 0.01	0.22 ± 0.02	n.d.	n.d.	0.35 ± 0.04
+1.0 μM ellipticine	0.18 ± 0.02	0.43 ± 0.04	n.d.	n.d.	0.61 ± 0.06
+10 μM ellipticine	4.02 ± 0.40	6.00 ± 0.60	n.d.	n.d.	10.02 ± 1.00
UKF-NB-3 (cisplatin)					
+0.1 μM ellipticine	0.03 ± 0.01	0.02 ± 0.01	n.d.	n.d.	0.05 ± 0.01
+1.0 μM ellipticine	0.06 ± 0.01	0.08 ± 0.01	n.d.	n.d.	0.14 ± 0.01
+10 μM ellipticine	18.4 ± 8.5	25.50 ± 2.54	n.d.	n.d.	43.99 ± 4.40
UKF-NB-4					
+0.1 μM ellipticine	0.04 ± 0.01	0.01 ± 0.01	n.d.	n.d.	0.05 ± 0.01
+1.0 μM ellipticine	0.20 ± 0.02	0.38 ± 0.04	n.d.	n.d.	0.58 ± 0.06
+1.0 μM ellipticine hypoxic conditions	0.18 ± 0.02	0.15 ± 0.02***	n.d.	n.d.	0.30 ± 0.02***
+10 μM ellipticine	5.40 ± 0.56	6.50 ± 0.81	0.27 ± 0.03	0.37 ± 0.05	12.54 ± 1.51
+10 μM ellipticine hypoxic conditions	1.86 ± 0.21***	3.74± 0.42***	0.26 ± 0.03	0.39 ± 0.03	6.25 ± 0.68***
UKF-NB-4 (Elli)					
+1.0 μM ellipticine	0.07 ± 0.01***	0.06 ± 0.01***	0.06 ± 0.01	n.d.	0.19 ± 0.02***
+1.0 μM ellipticine hypoxic conditions	0.08 ± 0.01***	0.06 ± 0.0***	0.04 ± 0.01***	n.d.	0.18 ± 0.02***
+10 μM ellipticine	1.99 ± 0.20***	1.43 ± 0.14***	0.15 ± 0.01***	0.02 ± 0.01***	3.60 ± 0.39***
+10 μM ellipticine hypoxic conditions	0.34 ± 0.03$^{***\Delta\Delta\Delta}$	0.24 ± 0.03$^{***\Delta\Delta\Delta}$	0.12 ± 0.01$^{***\Delta}$	0.02 ± 0.01**	0.72 ± 0.07$^{***\Delta\Delta\Delta}$
UKF-NB-4 (DOXO)					
+0.1 μM ellipticine	0.07 ± 0.01	0.10 ± 0.01	n.d.	n.d.	0.17 ± 0.02
+1.0 μM ellipticine	0.54 ± 0.05	1.02 ± 0.10	n.d.	n.d.	1.56 ± 0.16
+10 μM ellipticine	0.23 ± 0.20	1.22 ± 0.10	n.d.	n.d.	1.45 ± 0.14
UKF-NB-4 (cisplatin)					
+0.1 μM ellipticine	0.02 ± 0.01	0.03 ± 0.01	n.d.	n.d.	0.05 ± 0.01
+1.0 μM ellipticine	0.24 ± 0.02	0.42 ± 0.04	n.d.	n.d.	0.66 ± 0.07
+10 μM ellipticine	5.73 ± 0.58	9.66 ± 0.97	n.d.	n.d.	15.39 ± 1.50

Neuroblastoma cells were exposed to ellipticine for 48 h. DNA adducts were analyzed by the nuclease P1 version of the ^{32}P-postlabeling assay.
The data were analyzed statistically by Student's *t*-test. Values significantly different from individual parent cell lines cultivated under the standard conditions: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Values significantly different from the UKF-NB-4 (Elli) cell line cultivated under the standard conditions: $^{\Delta\Delta\Delta}P < 0.001$.
[a] RAL, relative adduct labeling; averages and S.D. of three experiments. n.d.: not detected (the detection limit of RAL was $1/10^{11}$ nucleotides).

To investigate whether the cytotoxic activity of ellipticine to neuroblastoma cells depends on the CYP- and/or peroxidase-catalyzed formation of ellipticine-DNA adducts, and if so, which is the relative contribution of each enzyme system, two experimental approaches were employed: (i) correlation of the IC_{50} values for ellipticine in each cell line with the levels of ellipticine-DNA adducts in the same line, and (ii) analysis of the effect of hypoxic cell culture conditions on ellipticine cytotoxicity and DNA adduct formation. Evaluation of ellipticine cytotoxicity under anaerobic conditions is also important, because such conditions mimic the environment typical for malignant tumors.

3.7. Cytotoxicity of and DNA adduct formation by ellipticine in neuroblastoma cells cultivated under hypoxic conditions

Growth inhibition and a cell cycle arrest are mediated by ellipticine in neuroblastoma cells even under hypoxic conditions of their cultivation. Ellipticine was less toxic to the cells grown under hypoxic conditions except to UKF-NB-3 cells (Table 1). The cell

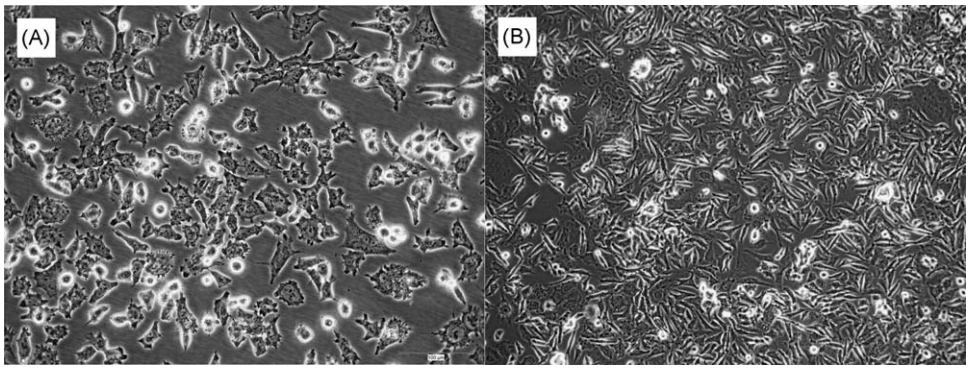

Fig. 6. Neuroblastoma cell lines UKF-NB-4 (A) and UKF-NB-4 resistant to ellipticine (B) (200-fold magnification).

growth rates under the hypoxic conditions were slower in IMR-32 and UKF-NB-4, and in this line resistant to ellipticine (see doubling times shown in Table 3). On the contrary, no changes in the doubling times were found in UKF-NB-3 cell lines (Table 3). The effects of ellipticine on the cell cycle of all original lines tested were not changed significantly by hypoxic conditions, but cultivation of UKF-NB-4 resistant to ellipticine under such conditions resulted in an arrest in S phase of the cell cycle (Fig. 3). Under hypoxic conditions the ellipticine-DNA adduct levels in neuroblastoma cells were lower, predominantly in IMR-32 and UKF-NB-4 cells (Table 2). In parental UKF-NB-4 cells and those resistant to ellipticine, the decrease in levels of ellipticine-DNA adducts by hypoxia was 5-fold and resulted from a decreased formation of adducts 1 and 2, but not of adducts 6 and 7 (Table 2). This finding shows that CYP enzymes, whose activities are dependent on oxygen, are predominantly responsible for formation of adducts 1 and 2 in these neuroblastoma cells, while generation of adducts 6 and 7 is mediated by peroxidases, which are oxygen independent.

Table 3
Doubling times[a] of neuroblastoma cells grown under standard and hypoxic conditions.

Neuroblastoma	Doubling times[a] (h)	
	Standard conditions	Hypoxic conditions
IMR-32	25.18 ± 0.22	$34.69 \pm 0.46^{***}$
UKF-NB-3	29.67 ± 0.28	30.11 ± 0.40
UKF-NB-4	26.68 ± 0.24	$32.56 \pm 0.75^{***}$
UKF-NB-4 (Elli)	$34.56 \pm 0.61^{\Delta\Delta\Delta}$	$44.30 \pm 1.60^{***\Delta\Delta\Delta}$

[a] Results shown are mean ± S.D. from data found of four experiments. The data were analyzed statistically by Student's *t*-test. Values of doubling times significantly different from those of the cells grown under standard conditions: $^{***}P < 0.001$. Values of doubling times significantly different from those of the UKF-NB-4 parent cell lines: $^{\Delta\Delta\Delta}P < 0.001$.

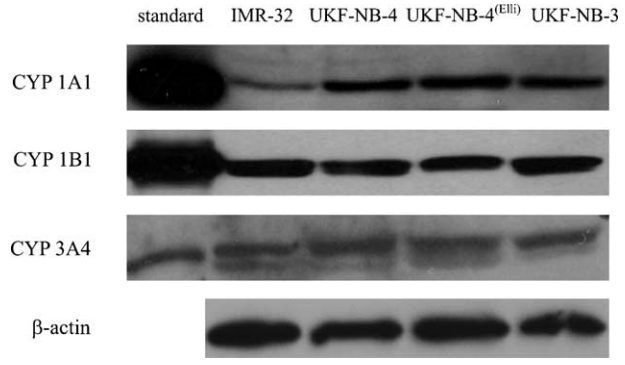

Fig. 7. Immunoblots of CYP1A1, CYP1B1, and CYP3A4 in neuroblastoma cell homogenates. Cell homogenates were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibodies as described in Material and Methods. Human CYP1A1, CYP1B1 and CYP3A4 were used as standards.

3.8. Correlation of cytotoxicity of ellipticine with DNA adduct formation

We analyzed the relationships between IC_{50} values for ellipticine and levels of ellipticine-DNA adducts. In the case of IMR-32 and UKF-NB-4 cells, high levels of DNA adducts correlated with ellipticine-induced cytotoxicity to these cells (Fig. 8). In contrast to these results, the ellipticine-induced cytotoxicity to the UKF-NB-3 cells showed no correlation to ellipticine-DNA adduct levels.

4. Discussion

The results of this study show that ellipticine is cytotoxic to human neuroblastoma cell lines (both to the parental IMR-32, UKF-NB-3, UKF-NB-4 lines and some of their sublines resistant to several antitumor drugs). In addition, the results shed light on the mechanism of ellipticine cytotoxicity to neuroblastoma cells. The mode of antitumor, cytotoxic and mutagenic action of ellipticine is considered to be based mainly on DNA damage such as intercalation into DNA [4,44], inhibition of topoisomerase II [4,14–16], and formation of covalent DNA adducts mediated by CYPs and peroxidases [3,19,21,24,45]. Intercalation of ellipticine into DNA and inhibition of topoisomerase II occur in all cell types irrespective of their metabolic capacity, because of the general chemical properties of this drug and its affinity to DNA and topoisomerase II protein [4,9,45]. However, the formation of ellipticine-DNA adducts, which is dependent on ellipticine activation by CYPs and peroxidases, has not yet been proven as a general mechanism. This ellipticine action was unambiguously found *in vitro*, using several CYP and peroxidase enzymes for ellipticine activation [3,17,19,21,24,45] and *in vivo* in rats and mice [8,22,24]. Ellipticine-DNA adducts were quantified also in three human cancer cell lines, breast adenocarcinoma MCF-7 cells [25], and the leukemia HL-60 and CCRF-CEM cells [26]. Because ellipticine derivatives target also neurological tumors, we investigated whether this mechanism occurs also in neuroblastoma cells. In addition, a role of this mechanism in ellipticine toxicity to these cancer cells was examined.

Using the ^{32}P-postlabeling assay we clearly demonstrated that ellipticine binds covalently to DNA of neuroblastoma cells; two major ellipticine-DNA adducts, identical with those formed by the CYP- and peroxidase-mediated ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine, were formed in all neuroblastoma cells tested in the study, including the line with induced resistance to ellipticine. These adducts are formed from two

Table 4
Expression of mRNA of CYP1A1, 1B1, 3A4, COX-1 and -2 in neuroblastoma cells.

Neuroblastoma	Δc_T[a]				
	CYP1A1	CYP1B1	CYP3A4	COX-1	COX-2
IMR-32	12.21 ± 0.13	5.19 ± 0.12	15.31 ± 0.73	12.99 ± 0.33	14.86 ± 0.28
UKF-NB-3	10.53 ± 0.15	3.35 ± 0.02	15.52 ± 0.67	4.63 ± 0.09	16.40 ± 0.11
UKF-NB-4	11.00 ± 0.48	6.25 ± 0.28	11.96 ± 0.19	9.04 ± 0.41	16.06 ± 0.30
UKF-NB-4 (Elli)	7.63 ± 0.08***	7.85 ± 0.35**	12.83 ± 0.13*	8.78 ± 0.02	7.80 ± 0.21***

[a] Δc_T was evaluated according to the following equations: $\Delta c_T = c_T$(target) − c_T(internal standard). Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. Results shown are mean ± S.D. from data found for three experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from the parent UKF-NB-4 cell line: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

reactive species, ellipticine-13-ylium and ellipticine-12-ylium (Fig. 1), which we had suggested earlier to react with one of the nucleophilic centers in the deoxyguanosine residue in DNA (e.g. the exocyclic amino group of guanine, Fig. 1) [19,21,23,24,41]. The low amount of each DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine or 12-hydroxyellipticine (Fig. 4), however, prevented their further structural characterization. Synthetic approaches are currently being followed in our laboratory to prepare authentic ellipticine-DNA adduct standards [41,46]. Besides these adducts, two additional adduct spots were found in UKF-NB-4 cells.

Hypoxia frequently occurs in tumors because of their fast growth and inadequate vascularization. It strongly correlates with advanced disease and poor outcome caused by chemoresistance. The covalent binding of ellipticine metabolites to DNA decreased when cells were cultivated and treated with ellipticine under hypoxic conditions. The importance of oxygen for the ellipticine-DNA adduct formation suggests that activation of ellipticine to species binding to DNA occurring in neuroblastoma cells is mediated by CYP enzymes. The observed effects of α-NF and ketoconazole upon NADPH-dependent ellipticine-DNA adduct formation in S9 fractions from neuroblastoma cells suggests that CYP1A1 and 3A4 are the relevant CYP enzymes for ellipticine activation. CYP3A4 and, to a lower extent CYP1A1/2 were also found to be the enzymes activating ellipticine in human and rat livers [3,18,19]. Human CYP1A1/2 and 1B1 also oxidize ellipticine to 9-hydroxy- and 7-hydroxyellipticine, which are detoxication products [19]. The expression levels and the activities of these enzymes are, therefore, crucial for the extent of covalent DNA modification by ellipticine in neuroblastoma cells. Indeed, all CYPs relevant for ellipticine metabolism (CYP1A1, 1B1 and 3A4) were found to be expressed both at mRNA and protein levels. Even though expression of peroxidases such as COX-1 and -2 or MPO have only been detected at mRNA levels not by Western blots, hydrogen peroxide stimulated the formation of ellipticine-DNA adducts in *in vitro* incubations with S9 fractions from neuroblastoma cells, particularly from UKF-NB-4 cell line. The pattern of the ellipticine-DNA adducts found in UKF-NB-4 cells also point to peroxidase activation in intact cells, because in addition to DNA adducts 1 and 2, two minor DNA adducts 6 and 7, the typical products of peroxidase activation [21], are formed in these cells. The objective of our future work is to analyze the expression of COX and MPO in more detail in UKF-NB-4 cells with more sensitive antibodies against these enzymes, in addition to investigating the expression of other peroxidases such as LPO, which was not analyzed here.

Table 5
DNA adduct formation by ellipticine activated by S9 fractions from neuroblastoma cells.

S9 fraction of cells	Levels of DNA adducts (RAL × 10^{-8})[a]		
	Adduct 1	Adduct 2	Total
IMR-32			
Without cofactor	0.093 ± 0.010	0.031 ± 0.010	0.124 ± 0.016
+NADPH	0.275 ± 0.021	0.300 ± 0.028	0.575 ± 0.052
+NADPH + α-NF	1.470 ± 0.131***	0.397 ± 0.031***	1.867 ± 0.165***
+NADPH + ketoconazol	0.110 ± 0.010***	n.d.	0.110 ± 0.010***
+H_2O_2	0.413 ± 0.040***	0.071 ± 0.009***	0.484 ± 0.050*
UKF-NB-3			
Without cofactor	0.113 ± 0.010	0.039 ± 0.010	0.152 ± 0.015
+NADPH	0.133 ± 0.011	0.150 ± 0.012	0.283 ± 0.025
+NADPH + α-NF	0.115 ± 0.012	0.090 ± 0.010***	0.205 ± 0.021**
+NADPH + ketoconazol	n.d.	n.d.	n.d.
+H_2O_2	0.230 ± 0.020***	0.068 ± 0.008**	0.298 ± 0.023
UKF-NB-4			
Without cofactor	0.040 ± 0.008	0.100 ± 0.010	0.140 ± 0.014
+NADPH	0.099 ± 0.010	0.190 ± 0.015	0.289 ± 0.026
+NADPH + α-NF	0.083 ± 0.010	0.207 ± 0.020	0.290 ± 0.026
+NADPH + ketoconazol	0.041 ± 0.007***	0.151 ± 0.016*	0.192 ± 0.020***
+H_2O_2	0.809 ± 0.081***	0.843 ± 0.060***	1.652 ± 0.150***

[a] Mean RAL (relative adduct labeling) of four determinations (duplicate analyses of two independent *in vitro* incubations). n.d.: not detected (the detection limit of RAL was $1/10^{11}$ nucleotides). The data were analyzed statistically by Student's *t*-test. Values significantly different from incubations with NADPH: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

A negative correlation was seen between the IC_{50} values for ellipticine and the formation of ellipticine-DNA adducts in IMR-32 and UKF-NB-4 cells. These findings suggest that the cytotoxic activity of ellipticine to these two neuroblastoma cell lines is a consequence of the formation of ellipticine-DNA adducts. The role of ellipticine-DNA adduct formation in cytotoxicity was further supported by the finding that a decrease in the levels of these adducts in IMR-32 and UKF-NB-4 cells under hypoxic conditions corresponded to a decrease in toxicity of ellipticine under these conditions. In addition, the cell growth rates under hypoxic conditions were slower in IMR-32, UKF-NB-4 and ellipticine-resistant UKF-NB-4 cell lines, which might also protect the cells from the cytotoxic effects of ellipticine.

A number of DNA-damaging agents have been shown to inhibit cell growth by arrest at the G1/S boundary of the cell cycle [47–49]. This cell arrest is thought to be an important cellular defense mechanism that prevents replication of damaged DNA. We found that exposure to ellipticine caused an accumulation of IMR-32 and UKF-NB-4 cells in S phase. It is tempting to speculate that the mechanism of the S phase delay is the inability of the DNA polymerase complex to replicate over ellipticine-induced DNA adducts. Namely, it has been shown that DNA damage blocks DNA replication and/or transcription by polymerase [49–51]. In addition, inhibition of DNA replication has also been implicated as a proximate initiator of apoptosis [50], which we found in neuroblastoma cells studied in this work. However, the question whether the ellipticine-modified DNA templates can really block DNA polymerase awaits further investigation.

The predominant role of covalent ellipticine-DNA binding in cytotoxicity has already been found previously in leukemia cell lines; the cytotoxicity of ellipticine in human promyelotic leukemia HL-60 cells and human leukemia CCRF-CEM cells

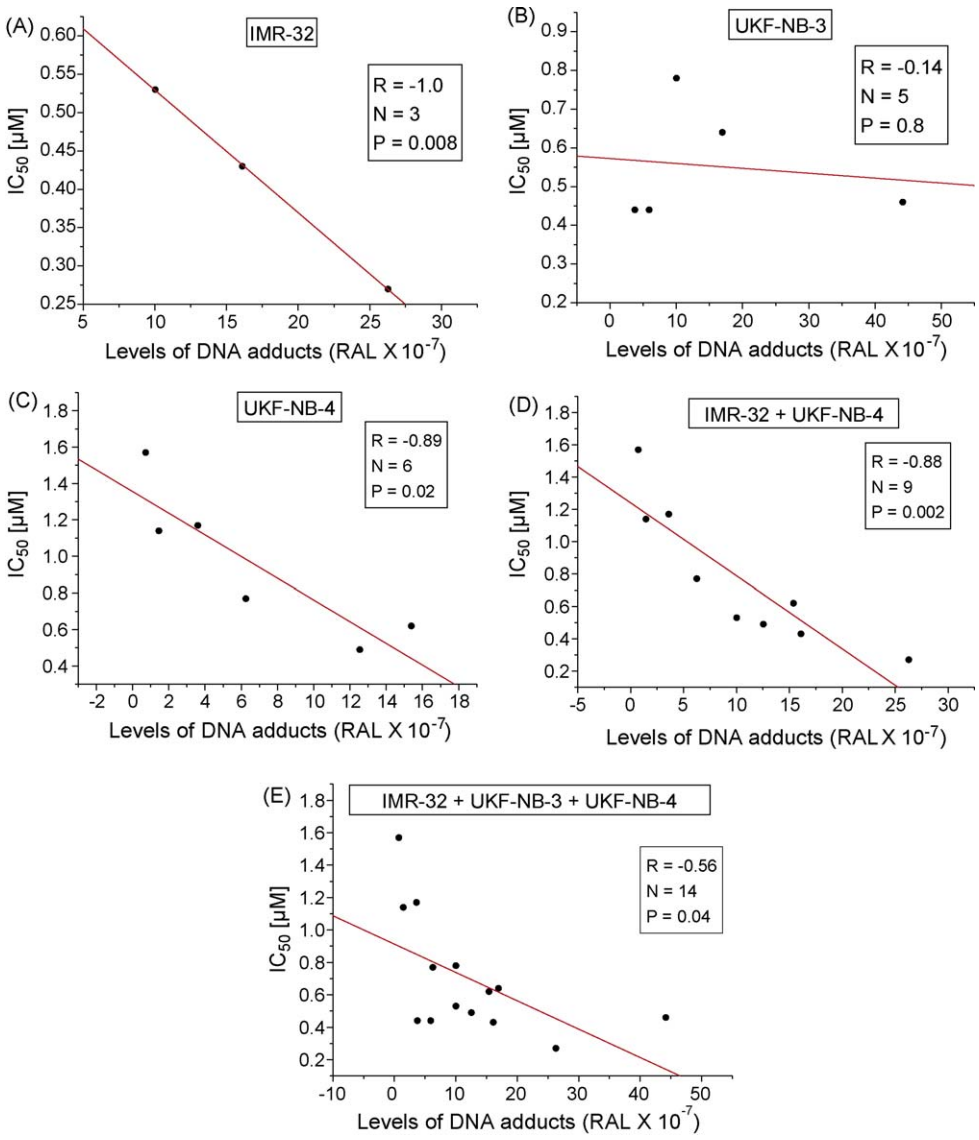

Fig. 8. Correlation of the formation of ellipticine-DNA adducts and IC_{50} values for ellipticine in IMR-32 (A), UKF-NB-3 (B), UKF-NB-4 (C), IMR-32 plus UKF-NB-4 (D) and IMR-32, UKF-NB-3 plus UKF-NB-4 cells (E). *R*, correlation coefficient; *N*, number of data points, *P*, level of significance.

correlated with the levels of ellipticine-derived DNA adducts generated in these cells [26]. The expression levels of CYP and peroxidase enzymes activating ellipticine in leukemia cells were found to be responsible for these results [26].

Likewise, Rekha and Sladek [52] demonstrated that the cytotoxic activity of ellipticine to MCF-7 cells depends on the levels of enzymes activating ellipticine to DNA-binding species, CYP enzymes in this case. The authors showed that MCF-7 cells treated with 3-methylcholanthrene transiently expressed elevated levels of CYP1A and were transiently much more sensitive to ellipticine. The DNA adducts we have observed in MCF-cells [25] might be responsible for the higher sensitivity observed by the above authors. Taken together, the data in the present work and those of previous studies [25,26,52] indicate that the activities and expression levels of CYP and peroxidase enzymes, which effectively activate ellipticine to metabolites forming DNA adducts, may be important factors in the specificity of ellipticine for acute myeloid leukemia, breast cancer and for some neuroblastomas.

However, a different response of the UKF-NB-3 cell line to ellipticine was found. Although ellipticine also induced apoptosis in this cell line, UKF-NB-3 cells were arrested in the G0/G1 and G2/M phase of the cell cycle. This finding indicates that mechanisms of ellipticine-mediated cytotoxicity in UKF-NB-3 cells are different from those in the other neuroblastoma cell lines tested in this study. Moreover, these mechanisms seem not to be associated with the covalent DNA damage caused by ellipticine, because no correlation was found between IC_{50} values for ellipticine and the formation of ellipticine-DNA adducts. Likewise, even though the levels of ellipticine-DNA adducts decreased in UKF-NB-3 cells under hypoxic conditions, no change in toxicity of ellipticine to these cells occurred. Therefore, acute ellipticine toxicity to UKF-NB-3 cells might be caused by its intercalation into DNA and/or inhibition of topoisomerase II, or by uncoupling mitochondrial oxidative phosphorylation and disrupting the cell's energy balance [10]. At the present time, we can only speculate on the mechanism explaining the different responses of UKF-NB-3 to ellipticine. The question whether they result from the biological heterogenicity of neuroblastoma cells, for example from their different genetic programs [29], remains to be answered.

Because drug resistance is a general feature of neuroblastoma, and arises in the majority of patients suffering from this cancer, we also investigated whether ellipticine is able to induce resistance in

neuroblastoma cells. The UKF-NB-4 cell line was used for such a study. The results demonstrate that ellipticine might, to some extent, induce resistance in these cells, but only after a long-term treatment with increasing concentrations of ellipticine. Resistance was accompanied by changes in cell morphology and 3-fold lower levels of ellipticine-DNA adducts. Even though the levels of mRNAs of CYP1A1 and COX-2 enzymes, by which formation of DNA adducts is mediated, were higher in the ellipticine-resistant UKF-NB-4 (Elli) cells, if cells of both lines were exposed to 10 μM ellipticine, mRNA levels of these enzymes were the same in the sensitive and the resistant line. Likewise, the expression of these enzymes at the protein level was not changed in the ellipticine-resistant line; COX-1 and -2 proteins were not detectable by Western blotting in either cell line. Levels of CYP3A4 protein expression were, however, slightly decreased in the ellipticine-resistant UKF-NB-4 line (Fig. 7), which might be responsible for a decrease in DNA adduct levels in these cells. Another mechanism might result from a shift of the ellipticine binding to CYP enzymes, from its binding as a CYP substrate (a type I ligand) to that as a heme ligand (a type II ligand) in the ellipticine-resistant UKF-NB-4 line. It has been found that ellipticine may, depending on several conditions such as pH, temperature [53] and the presence of endogenous CYP substrates [54], bind to some CYPs including the major enzyme activating this drug, CYP3A, as a heme ligand [53]. Because the heme ligands inhibit the initiation of the CYP reaction cycle, by preventing oxygen binding to the heme, ellipticine might in this case decrease its own activation by this mechanism [53].

The resistance factor of this cell line to ellipticine was lower (2.7) than that of the doxorubicin-resistant UKF-NB-4 cell line to doxorubicin (5.4). Likewise, neuroblastoma UKF-NB-3 and UKF-NB-4 cells resistant to vincristine and cisplatin were only slightly resistant to ellipticine. Even though the IC_{50} values for vincristine and cisplatin in these cells were not determined in this study, it is noteworthy that resistance of another neuroblastoma cell line, UKF-NB-2, to vincristine decreased toxicity of this drug significantly (resistance factor of this cell line to vincristine was 274) [31]. Moreover, resistance to doxorubicin, vincristine and cisplatin was induced during much shorter cultivation period with these drugs [29,31]. In addition, the IC_{50} value for ellipticine in the ellipticine-resistant UKF-NB-4 cell line, even under the hypoxic conditions, is still one order of magnitude lower than the ellipticine levels in blood of mice 4 h after their p.o. treatment with ellipticine in a tolerable dose [55]. The resistance mechanisms to ellipticine will be investigated in our laboratories, by analyzing the changes in the genetic programs in this cell line. Preliminary experiments suggest that ellipticine resistance is not dependent on P-glycoprotein expression, which is the case in the UKF-NB-4 cell line resistant to doxorubicin [29].

Collectively, the results presented in this paper are the first report demonstrating the cytotoxicity of ellipticine and formation of ellipticine-DNA adducts in human neuroblastoma cells. One of the most important results of this study is the finding that formation of ellipticine-DNA adducts is the predominant mechanism leading to cytotoxicity. Another important result of the study is finding that neuroblastoma cells can become resistant to ellipticine only after prolonged treatment and to a much lower extent than to doxorubicin or vincristine [31]. This finding is a promising result that might lead to the development of new neuroblastoma therapies, substituting or combining current anticancer drugs with ellipticine or its derivatives.

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