ORIGINAL ARTICLE

Cytotoxicity of and DNA adduct formation by ellipticine in human U87MG glioblastoma cancer cells

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Abstract

OBJECTIVES: Ellipticine is a potent antineoplastic agent exhibiting multiple mechanisms of action with promising brain tumor specificity. This anticancer agent should be considered a pro-drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its cytochrome P450 (CYP) - and/or peroxidase-mediated activation to species forming covalent DNA adducts. Ellipticine can also act as an inhibitor or inducer of biotransformation enzymes, thereby modulating its own metabolism leading to its genotoxic and pharmacological effects. The toxicity of ellipticine to U87MG glioblastoma cells and mechanisms of its action to these cells are aims of this study.
METHODS: Ellipticine metabolites formed in U87MG cells were analyzed using HPLC. Covalent DNA modifications by ellipticine were detected by ^{32}P-postlabeling. CYP enzyme expression was examined by QPCR and Western blot.
RESULTS: U87MG glioblastoma cell proliferation was efficiently inhibited by ellipticine. This effect might be associated with formation of two covalent ellipticine-derived DNA adducts, identical to those formed by 13-hydroxy- and 12-hydroxyellipticine, the ellipticine metabolites generated by CYP1A1, 1B1 and 3A4, lactoperoxidase and cyclooxygenase 1, the enzymes expressed in U87MG cells. Moreover, by inducing CYP1B1, 3A4 and 1A1 enzymes in U87MG cells, ellipticine increases its own enzymatic activation, thereby enhancing its own genotoxic and pharmacological potential in these cells. Ellipticine concentration used for U87MG cell treatment is extremely important for its pharmacological effects, as its metabolite profiles differed substantially predicting ellipticine to be either detoxified or activated.
CONCLUSION: The results found in this study are the first report showing cytotoxicity and DNA adduct formation by ellipticine in glioblastomas.

Abbreviations:

ATCC	– American Type Culture Collection
cDNA	– complementary DNA
COX-1	– cyclooxygenase I
CYP	– cytochrome P450
EMEM	– Eagle`s Minimum Essential Medium
FBS	– fetal bovine serum
HPLC	– high performance liquid chromatography
HRP	– horseradish peroxidise
IC_{50}	– concentrations of ellipticine causing death of half cells
IU	– international unit
LPO	– lactoperoxidase
MPO	– myeloperoxidase
PBS	– phosphate buffer saline
PCR	– polymerase chain reaction
POLR2	– polymerase (RNA) II (DNA directed) polypeptide A
PVDF	– polyvinylidene fluoride
SDS-PAGE	– sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M.	– standard error medium
QPCR	– quantitative polymerase chain reaction

INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Figure 1), an alkaloid isolated from *Apocyanaceae* 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 and anti-HIV activities (for a summary see Stiborova *et al.* 2001).

Ellipticine has been reported to arrest cell cycle progression by regulating the expression of cyclin B1 and Cdc2 as well as phosphorylation of Cdc2 (Kuo *et al.* 2005a,b), 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 (Kuo *et al.* 2005a,b, 2006), an increase of wild-type p53, the rescue of mutant p53 activity and the initiation of the mitochondrial apoptosis pathway (Garbett & Graves, 2004; Kuo *et al.* 2005a,b, 2006). Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines (Ohashi *et al.* 1995, Sugikawa *et al.* 1999), and this correlates with their cytotoxic activity. However, the precise molecular mechanism responsible for these effects has not been explained yet. Chemotherapy-induced cell cycle arrest was shown to result from DNA damage caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of its antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA, and (ii) inhibition of DNA topoisomerase II activity (Auclair, 1987, Garbett & Graves, 2004; Stiborova *et al.* 2006).

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 (Stiborova *et al.* 2001, 2003a,b, 2004, 2007a,b, 2008; Poljakova *et al.* 2006), 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, which are excreted (7-hydroxy- and 9-hydroxyellipticine) or which form deoxyguanosine adducts in DNA (13-hydroxy- and 12-hydroxyellipticine, the latter one formed also by Polonowski rearrangement from another metabolite, ellipticine-N^2-oxide) (***Figure 1***) (Stiborova *et al.* 2001, 2003a,b, 2004, 2007a). Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidase (LPO), human

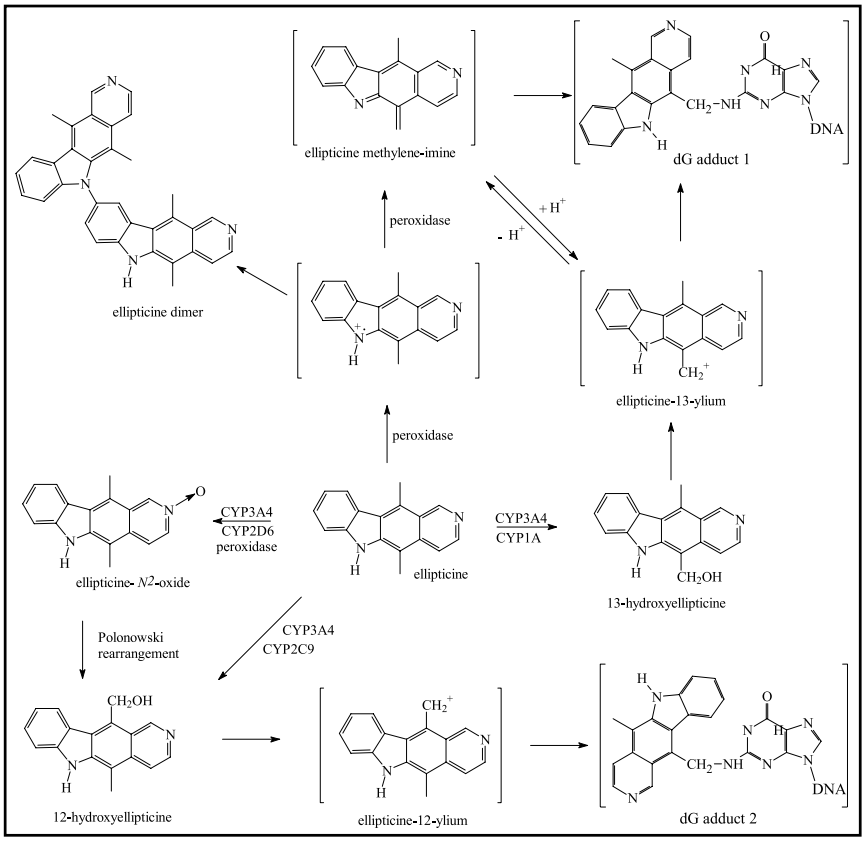


Figure1. Metabolism of ellipticine by peroxidases and human CYPs showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

myeloperoxidase (MPO) and horseradish peroxidase (HRP) efficiently generated ellipticine-derived DNA adducts (***Figure 1***) (Poljakova *et al.* 2006, Stiborova *et al.* 2007a). CYP- and/or peroxidase-mediated ellipticine-DNA adducts were detected also in rats and mice *in vivo* (Stiborova *et al.* 2003a, 2007b, 2008). The same DNA adducts were also detected in the culture of cells expressing enzymes activating ellipticine (CYP1A1, 1B1, 3A4, COX-1 and MPO), such as human breast adenocarcinoma MCF-7 cells (Borek-Dohalska *et al.* 2004), leukemia HL-60 and CCRF-CEM cells (Poljakova *et al.* 2007), neuroblastoma cell lines (Poljakova *et al.* 2009) and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (Frei *et al.* 2002). 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.

Recently, ellipticine was also shown to be brain tumor specific, by screening the National Cancer Institute tumor panel (Shi *et al.* 1998a,b). As glioblastomas are highly aggressive brain tumors hardly treatable with conventional therapies and often resistant to chemotherapy and/or radiotherapy with a very poor prognosis (Penas-Prado **&** Gilbert, 2007; Sathornsumetee *et al.* 2007), the aim of this study was to investigate the effects of ellipticine on a U87MG glioblastoma cell line and the mechanisms of ellipticine`s action in these cancer cells.

MATERIALS AND METHODS

U87MG cell line was purchased from ATCC (Manassas, VA, USA). Cells were cultured in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.6 mg/mL glutamine, 200 IU/mL penicillin, 200 IU/mL streptomycin, and 0.1 mg/mL gentamicin (PAA Laboratories, Pasching, Austria) in humidified 5% CO_2 at 37°C. IC_{50} were determined after 72 hours of ellipticine treatment using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, France) according to manufacturer`s instructions. Ellipticine (Sigma, St. Louis, MO, USA) and its metabolites were extracted with ethyl acetate from pellets of harvested cells resuspended in PBS. Organic phase was collected and evaporated to dryness, then resuspended in methanol and separated by HPLC as described earlier (Stiborova *et al.* 2004). Procedures for analysis of ellipticine-derived DNA adducts by the ^{32}P-postlabeling assay were performed as described by (Stiborova *et al.* 2001, 2004). RNA was extracted with Trizol (Sigma) and was transcribed into cDNA using high capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). mRNA contents were quantified using real-time quantitative polymerase chain reaction (QPCR) using the ABI7300 FAM™ PCR detector with the commercially available unlabeled PCR primers POLR2 as a reference internal standard gene, CYP1A1, 1B1 and 3A4 (Generi Biotech, Hradec Kralove, Czech Republic). Relative levels of mRNA gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method described previously by (Livak **&** Schmittgen, 2001). Enzyme expression was determined by Western blot after 72 hours of 1 μM ellipticine treatment. Briefly: cells were detached with trypsin, lysed on ice with RIPA buffer enriched with Protease Cocktail Inhibitor (Roche, Basel, Switzerland). Human CYPs in Supersomes™ (Gentest Corp., Woburn, MA, USA) were used as standards. Proteins were separated using SDS-PAGE (10% polyacrylamide) and transferred to PVDF membranes. Blots were probed with appropriate antibodies (CYP1A1 – Millipore, Billerica, MA, USA; CYP1B1, LPO, COX-1 – Abcam, Cambridge, MA USA; CYP3A4; - AbD Serotec, Oxford, UK) followed by HRP-conjugated secondary antibodies (BioRad, Hercules, CA, USA). Immun-Star™ HRP Substrate Kit (BioRad) was used to visualize proteins and band intensities were quantified by Elfoman software (Krizkova *et al.* 2008).

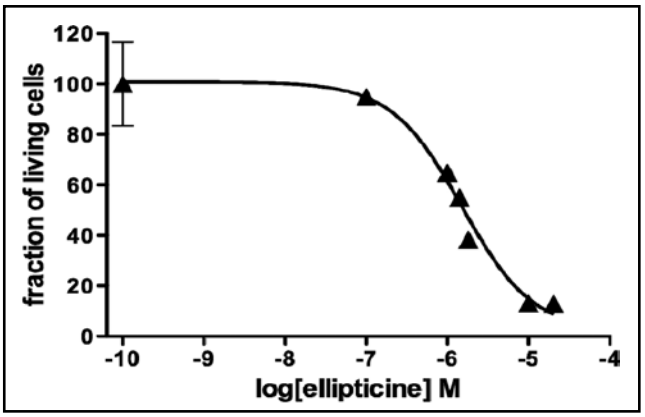


Figure 2. Proliferation assay. U87MG cells were treated 72 hours in 2% FBS medium with either solvent (dimethyl sulfoxide) or ellipticine (0.1 - 20 μM). Proliferation was expressed as the percentage of living cells after ellipticine treatment compared to untreated cells grown in the presence of solvent considered as the 100%. IC_{50} were determined using GraphPad Prism software, non-linear sigmoidal dose-response regression.

RESULTS

To determine the cytotoxicity of ellipticine to human glioblastomas, U87MG glioblastoma cells were treated with increasing concentrations of ellipticine. Ellipticine inhibited proliferation of a U87MG glioblastoma cell line with IC_{50} of 1.48 ± 0.08 μM (***Figure 2***).

Recently, we have shown that ellipticine forms covalent DNA adducts in various tissues including rat brain (***Figure 3a***), after being enzymatically activated with CYPs or peroxidases (Stiborova *et al.* 2001, 2003a,b, 2004, 2007a,b, 2008; Poljakova *et al.* 2006). Here, we tested whether such DNA adducts are also generated by ellipticine in DNA of U87MG glioblastoma cells. The U87MG cells were treated with 0.5, 1 and 10 μM ellipticine for 72 hours. Using the nuclease P1 version of ^{32}P-postlabeling assay, which was found to be suit-

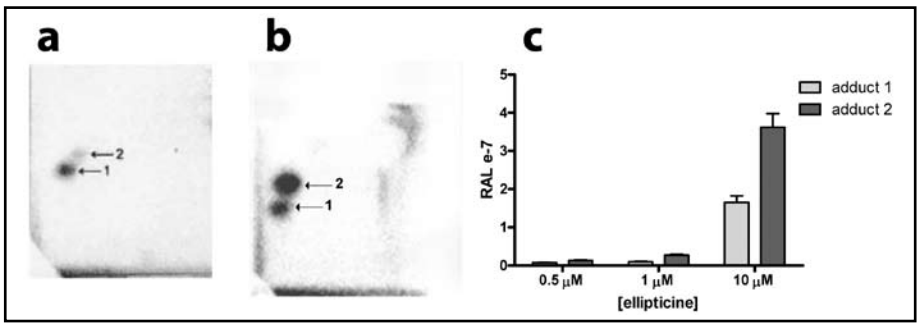
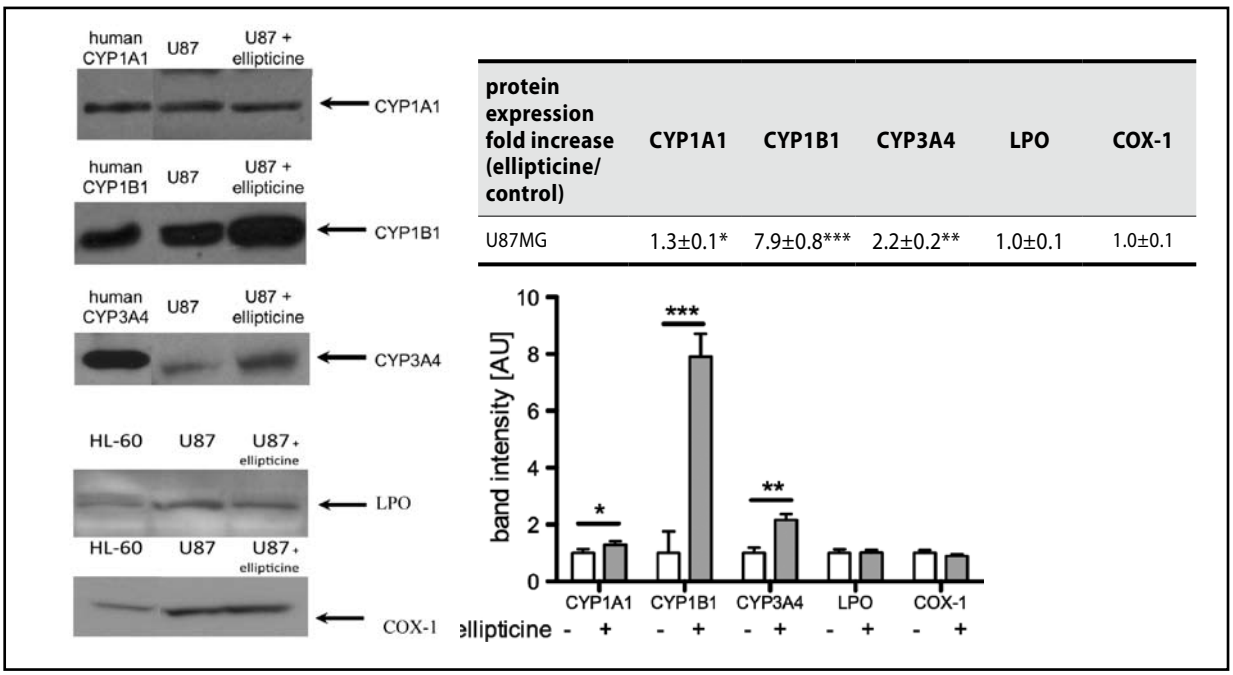


Figure 3. Autoradiograph of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from (*a*) brain of rats treated with 40 mg ellipticine per kg body weight (Stiborová *et al.* 2003a), (*b*) U87MG cells treated with 10 M ellipticine. (*c*) Quantification of the ellipticine-DNA adduct levels generated in U87MG cells treated with ellipticine for 72 hours. Analyses were performed by the nuclease P1 version of the assay (Stiborova *et al.* 2001; Stiborova *et al.* 2003a,b). Film exposure was 4 hr at -80°C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right). Data are represented as means ± S.E.M., *n* = *3*.

Figure 4 (below). The CYP1A1, 1B1, 3A4, LPO and COX-1 protein expression in U87MG cells treated with 1 μM ellipticine in 2% FBS medium for 72 hours. Total protein extracts were resolved by SDS-PAGE, transferred to PVDF membranes and probed with anti-CYP antibodies. GAPDH was used as a loading control. A representative blot is shown and the graph displays means ± S.E.M of relative band intensities quantified using Elfoman software (*n*=3). The data were analyzed statistically by Student's *t*-test. Values significantly different from cells cultivated without ellipticine: *P<0.05, **P<0.01, ***P<0.001.



protein expression fold increase (ellipticine/ control)	CYP1A1	CYP1B1	CYP3A4	LPO	COX-1
U87MG	1.3±0.1*	7.9±0.8***	2.2±0.2**	1.0±0.1	1.0±0.1

able to detect and quantify DNA adducts formed by ellipticine (Stiborova *et al.* 2001, 2003a,b, 2004, 2007a,b, 2008; Poljakova *et al.* 2006), ellipticine-derived adducts were detected in the DNA of these cells (***Figure 3b,c***). Two major ellipticine-DNA adducts (spots 1 and 2 in Figure 3a,b) were formed in these cells. Both these adducts were found to be generated from 13-hydroxy- and 12-hydroxyellipticine (Stiborova *et al.* 2004, 2007a) as confirmed by cochromatographic analysis using TLC and HPLC (data not published). Ellipticine-DNA adduct levels were dose dependent in U87MG cells with an overproportional increase between 1 μM and 10 μM ellipticine (Figure 3c). No adducts were detected in DNA of control cells treated with solvent only.

Dutheil and co-workers found that twenty four CYP enzymes (CYP1, CYP2 and CYP3 families and CYP46A1) are expressed in human brain tissues (Dutheil *et al.* 2009). From the CYPs known to metabolize ellipticine, expression of CYP1B1 and 1A1 were the most important regarding mRNA levels (Dutheil *et al.* 2009). Therefore, expression of these CYP enzymes as well as that of another CYP enzyme that is crucial for ellipticine metabolic activation, CYP3A4, was analyzed in U87MG glioblastoma cells. Using Western blot analysis with polyclonal antibodies raised against CYP1A1, 1B1 and 3A4, the protein expression of these enzymes was detected in U87MG cells (***Figure 4***). Beside these CYP enzymes, peroxidases such as lactoperoxidase (LPO) and cyclooxygenase I (COX-1) were found to be expressed in U87MG cells (Figure 4).

Since the expression of several CYP enzymes is known to be increased by ellipticine (Aimova *et al.* 2007), we examined whether ellipticine might be capable of inducing CYP enzymes expressed in U87MG cells. Western blots with antibodies against CYP1A1, 1B1 and 3A4 (see above) showed that the expression of

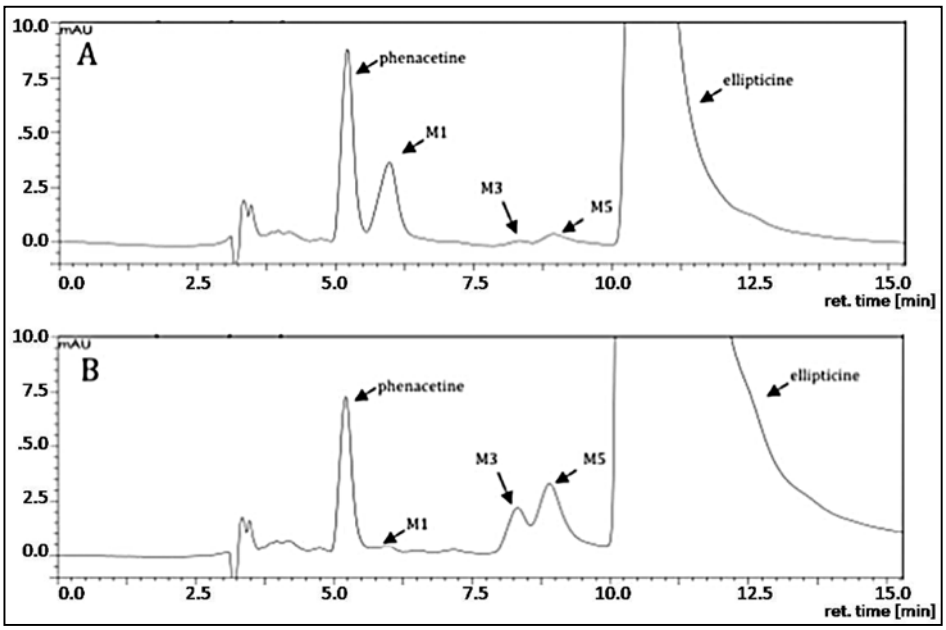


Figure 5: HPLC separation of ellipticine metabolites formed in U87MG cells. Cells were treated with 1 μM (A) and 10 μM (B) ellipticine for 72 hours in 2 % FBS containing EMEM. HPLC column: C18, 250 x 4.6 mm, 5 μm (Beckman, Fullerton, CA, USA), mobile phase: 64% methanol and 36% of 0.005 M heptane sulfonic acid and 0.032 M acetic acid in distilled water, detection wavelength: 296 nm.

CYP1B1 and 3A4 was induced in U87MG cells treated with ellipticine (Figure 4). The expression of CYP1A1 was also increased, but to a lower extent (***Figure 4***). Besides the effect of ellipticine on protein expression of these enzymes, that on mRNA expression of CYPs was also investigated. Total RNA was isolated from frozen cells and the relative amounts of *CYP1A1, 1B1* and *3A* mRNAs were measured by real-time QPCR. As shown in ***Table 1***, expression of mRNAs of these enzymes was also induced by ellipticine, but the increase in expression of *CYP1B1* mRNA was not statistically significant (Figure 4).

In contrast to induction of CYP enzymes by ellipticine, the protein expression levels of both peroxidases tested in this study, COX-1 and LPO, in U87MG cells were not affected by ellipticine.

Because of the induction potential of ellipticine to increase expression of CYP enzymes tested in this study (CYP1A1, 1B1 and 3A4), the enzymes responsible both for ellipticine activation and detoxication (Stiborova *et al.* 2001, 2003a, 2004, 2008), the effect of ellipticine on its own metabolism in U87MG cells was examined. HPLC with UV detection was used to separate ellipticine metabolites (***Figure 5***). When low concentrations of ellipticine (1 μM) were used for the treatment of U87MG cells, detoxifying ellipticine metabolite 9-hydroxyellipticine (peak M1 in Figure 5) was predominantly formed in these cells. On the contrary, when U87MG cells were treated with 10 μM ellipticine, pattern of detoxification and activation ellipticine metabolites changed significantly. Namely, 13-hydroxyellipticine (peak M3 in Figure 5) and ellipticine-N^2-oxide (peak M5 in Figure 5), the ellipticine metabolites responsible for formation of covalent ellipticine-derived DNA adducts (see Figure 1), were formed as the major products of ellipticine oxidation in U87MG cells.

Table 1. Induction of *CYP1A1, 1B1* and *3A4* mRNAs in U87MG cells after treatment with 1 μM ellipticine for 72 hours in 2 % FBS containing EMEM medium. Values are means ± S.E.M. of 5 experiments. The data were analyzed statistically by Student's *t*-test. Values significantly different from control cells cultivated without ellipticine: *P<0.05.

mRNA fold increase (ellipticine/control)	CYP1A1	CYP1B1	CYP3A4
U87MG	3.98 ± 1.58*	1.77 ± 0.49	8.37 ± 5.50*

DISCUSSION

The results of this study show that U87MG glioblastoma cell line is sensitive to ellipticine. In addition, the results shed some light on the mechanism of ellipticine cytotoxicity to these glioblastoma cells.

Glioblastomas are highly aggressive brain tumors hardly treatable with conventional therapies and often resistant to chemotherapy and/or radiotherapy with a very poor prognosis (Penas-Prado & Gilbert, 2007; Sathornsumetee *et al.* 2007). Ellipticine is an anticancer drug exhibiting a high potential to inhibit tumor growth in diverse cancers (Mondesir *et al.* 1985; Rouesse *et al.* 1985; Somers *et al.* 1985; Arguello *et al.* 1998; Acton *et al.* 1994). As it has been shown to be brain-tumor specific (Shi *et al.* 1998a,b), here we investigated its possible benefits in such aggressive brain tumors as glioblastomas. We showed that ellipticine significantly inhibited U87MG glioblastoma proliferation *in vitro* with IC_{50} in micromolar ranges, which is comparable to other ellipticine-sensitive cancer cell lines. Because ellipticine is known to exhibit multiple mechanisms of action (Auclair, 1987), here we evaluated the mechanisms that might be implicated in U87MG growth inhibition.

The mode of antitumor, cytotoxic and mutagenic action of ellipticine is considered to be based mainly on DNA damage such as intercalation into DNA (Auclair, 1987; Chu and Hsu, 1992), inhibition of topoisomerase II (Auclair, 1987; Froelich-Ammon *et al.* 1995), and formation of covalent DNA adducts mediated by CYPs and peroxidases (Stiborova *et al.* 2001, 2003a,b, 2004, 2006, 2007a,b, 2008; Poljakova *et al.* 2006). 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 (Auclair, 1987, Garbett & Graves, 2004; Stiborova *et al.* 2006). 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 type of ellipticine mode of action was unambiguously found *in vitro*, using several CYP and peroxidase enzymes for ellipticine activation (Stiborova *et al.* 2001, 2003b, 2004, 2007a; Poljakova *et al.* 2006) and *in vivo* in rats and mice (Stiborova *et al.* 2003a, 2007b, 2008). Ellipticine-DNA adducts were quantified also in several human cancer cell lines, such as breast adenocarcinoma MCF-7 cells (Borek-Dohalska *et al.* 2004), leukemia HL-60 and CCRF-CEM cells (Poljakova *et al.* 2007) and neuroblastoma cell lines (Poljakova *et al.* 2009), being proved to be the predominant mechanism of ellipticine action in these cancer cells. In this study we confirmed such DNA adducts in other human cancer cells, in a U87MG glioblastoma cell line. Because the covalent ellipticine-derived DNA adducts are also generated in brain tissues *in vivo*, in rats treated with ellipticine (Stiborova *et al.* 2003a, 2007b) (see also Figure 3b), ellipticine and/or its metabolites probably overcome the blood-brain barrier.

Using the ^{32}P-postlabeling assay we clearly demonstrated that ellipticine binds covalently to DNA of U87MG gliobastoma 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 these cells. These adducts are formed from two reactive species, ellipticine-13-ylium and ellipticine-12-ylium (Figure 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, Figure 1). The low amount of each DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine or 12-hydroxyellipticine, however, prevented their further structural characterization. Synthetic approaches are currently being followed in our laboratory to prepare authentic ellipticine-DNA adduct standards (Dracinsky *et al.* 2007; Moserova *et al.* 2008).

Ellipticine concentrations used for the treatment of U87MG cells appeared to be highly important; the metabolite profiles differed substantially, predicting ellipticine to be either detoxified or activated when U87MG cells were exposed to different ellipticine concentrations. Moreover, ellipticine influences its own metabolism *via* induction of CYP enzymes metabolizing this anticancer drug. Indeed, an overproportional increase in levels of ellipticine-derived DNA adducts in U87MG cells treated with 1 μM and 10 μM ellipticine was found in these cells. Such finding might follow from ellipticine-mediated induction of CYP3A4, the enzyme producing higher levels of activation ellipticine metabolites (13-hydroxyellipticine and ellipticine-N^2-oxide), found in this study. Therefore, ellipticine dosage for cancer treatment should be considered carefully.

Induction of *CYP1A1, 1B1 and 3A4* mRNAs by ellipticine was different from induction of proteins of these enzymes. Similar discrepancies between induction of mRNA of several CYPs and protein levels were observed also in rats treated with ellipticine (Aimova *et al.* 2007) and for other compounds as found by several authors (Chen *et al.* 1998, Iba *et al.* 1999, 2000; Dickins, 2004). It has been reported that some inducers might prolong half-lives of mRNAs, while others increase their transcription.

Collectively, the results presented in this paper are the first report demonstrating sensitivity of a U87MG glioblastoma cell line to ellipticine. Moreover, we proved that ellipticine binds covalently to DNA of U87MG cells, forming the two deoxyguanosine adducts by its activation to 13-hydroxy and ellipticine-N^2-oxide that rearranges to 12-hydroxyellipticine in DNA of U87MG cells and that this feature might be at least one of the causes of the U87MG sensitivity to ellipticine treatment.

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