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ORIGINAL ARTICLE

Cytotoxicity of and DNA adduct formation by ellipticine and its micellar form in human leukemia cells *in vitro*

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Abstract

OBJECTIVES: The improvements of cancer treatment are the major challenge in oncology research. Nanocarriers are one of the promising approaches to selectively target tumor cells, frequently leading to improve drug therapeutic index. Ellipticine is an anticancer agent that functions through multiple mechanisms. Here, the toxic effects of an anticancer drug ellipticine encapsulated in a micellar nanotransporter and free ellipticine on human HL-60 leukemia cells and formation of ellipticine-derived DNA adducts by both forms of the drug in these cells were investigated. **METHODS:** The toxicity of modified ellipticine on cells was compared to that of free ellipticine using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide cytotoxicity assay. ^{32}P-postlabeling was utilized to determine ellipticine-DNA adducts in treated cells. **RESULTS:** The comparison of efficiencies of free ellipticine and ellipticine-micelles [the poly(ethylene oxide)-block-poly(allyl glycidyl ether) *block* copolymer] to form ellipticine-derived DNA adducts in leukemia HL-60 cells and to act as cytotoxic agent on these cells was performed. Exposure of HL-60 cells to ellipticine in micelles resulted in formation of ellipticine-DNA adducts and caused the cytotoxic effect on these cells. The influence of ellipticine in micelles on HL-60 cells was very similar to that of free ellipticine. The ellipticine half maximal inhibition concentration was determined as 1.3±0.3 μmol.L^{-1} and 1.4±0.3 μmol.L^{-1} for ellipticine and ellipticine in micelles, respectively. Likewise, the levels of ellipticine-DNA adducts generated in HL-60 cells by both forms of ellipticine were analogous. **CONCLUSION:** The results found in this work demonstrate similar cytotoxicity and DNA-damaging effects of ellipticine and its micellar form on leukemia HL-60 cells *in vitro*.

Abbreviations

CYP	- cytochrome P450
DMSO	- dimethyl sulfoxide
EPR	- enhanced permeation and retention
IC_{50}	- half maximal inhibition concentration
IMDM	- Iscove's modified Dulbecco's medium
MDDS	- micellar drug delivery system
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
PAGE-PEO	- poly(ethylene oxide)-*block*-poly(allyl glycidyl ether)
RAL	- relative adduct labeling
TLC	- thin layer chromatography

INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Figure 1) and its derivatives are efficient anticancer compounds that function through multiple mechanisms of their action [for a summary see (Auclair 1987; Stiborova *et al.* 2001, 2006, 2011; Garbett & Graves 2004; Kizek *et al.* 2012; Stiborova & Frei 2014)]. Ellipticine was found (*i*) to inhibit cell growth, (*ii*) to arrest cell cycle, and (*iii*) to induce the mitochondria-dependent apoptotic processes in cells [for a summary see (Stiborova *et al.* 2011; Kizek *et al.* 2012; Stiborova & Frei 2014)]. The predominant mechanisms responsible for these ellipticine's biological effects were suggested to be (*i*) intercalation into DNA and (*ii*) inhibition of topoisomerase II (Auclair 1985; Stiborova *et al.* 2001, 2006, 2011; Garbett & Graves 2004; Kizek *et al.* 2012; Stiborova & Frei 2014; Tmejova *et al.* 2014). Further, we showed that this antitumor agent forms covalent DNA adducts after its enzymatic activation with cytochromes P450 (CYP) and peroxidases (Stiborova *et al.* 2001, 2004, 2006, 2007a, 2007b, 2011, 2012, 2013; Kotrbova *et al.* 2011; Kizek *et al.* 2012; Stiborova & Frei 2014), suggesting an additional DNA-damaging effect of ellipticine.

Of the CYP enzymes investigated, human microsomal CYP3A4 followed by CYP1A1 and 1B1 are the most active enzymes oxidizing ellipticine to 12-hydroxy- and 13-hydroxyellipticine, whose formation is influenced by another protein expressed in the membrane of endoplasmic reticulum, cytochrome b_5. These reactive metabolites dissociate to ellipticine-12-ylium and ellipticine-13-ylium which form two major covalent DNA adducts (Figure 1) (Stiborova *et al.* 2001, 2004, 2007a, 2007b, 2012, 2013; Moserova *et al.* 2008; Kotrbova *et al.* 2012; Stiborova & Frei 2014).

The same ellipticine-derived DNA adducts that were found in *in vitro* incubations of ellipticine with DNA and enzymes activating this drug, were generated also *in vivo*, in several tissues of mice and rats exposed to ellipticine, in several cancer cell lines and in DNA of rat mammary adenocarcinoma *in vivo* (Stiborova *et al.* 2003, 2007b, 2008, 2010, 2011, 2014b; Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007, 2009, 2011, 2013; Martinkova *et al.* 2009).

There are, however, several phenomena that can cause a limited usage of ellipticine and/or its limited anticancer efficiencies. This antineoplastic agent exhibits also severe adverse toxic effects, including nephrotoxicity, hemolysis, xerostomia, hypertension, nausea and vomiting [for a review see (Stiborova & Frei 2014)]. The question, therefore, arises how to eliminate its toxic side effects as well as to utilize novel knowledge on their bio-activation in cancer cells to potentiate their anticancer efficiencies in these cancer cells. Hence, the studies of our laboratory are targeted on development of efficient and reliable methods for targeted delivery of ellipticine (and/or other anticancer drugs) as well as on preparation of this drug in the forms that exhibit lower side effects and leads to an increase in their anticancer effects. One of the aims is to develop nanocarriers that will contain this drug.

In our former study, we utilized the poly(ethylene oxide)-block-poly(allyl glycidyl ether) (PAGE-PEO) block copolymer (P 1191 nanoparticles) (Hruby *et al.* 2005) to prepare a micellar form of ellipticine and investigated its biodistribution among the tissues of a model organism, Wistar rats (Stiborova *et al.* 2014a), which might, to some extent, mimic the fate of ellipticine in humans (Stiborova *et al.* 2003, 2007b, 2011; Stiborova & Frei 2014). The results found in this former work demonstrated that treatment of rats with ellipticine in micelles resulted in its biodistribution in rats and in formation of ellipticine-derived DNA adducts, and suggest that a gradual release of ellipticine from its micellar form might produce the enhanced permeation and retention (EPR) effect of this ellipticine-micellar delivery system (Stiborova *et al.* 2014a).

In this work, we used the same block copolymer (P 1191 nanoparticles) (Hruby *et al.* 2005), from which we prepared a micellar form of ellipticine (Stiborova *et al.* 2014a), and examined its effects on human leukemia HL-60 cells that were found previously to be sensitive to ellipticine (Poljakova *et al.* 2007). The formation of ellipticine-derived DNA adducts as the biological endpoint of the pharmacological and genotoxic effects of this drug, mediated by free ellipticine and its micellar form, measured with the ^{32}P-postlabeling method, was used to determine their entrance into the HL-60 cells *in vitro*. Further, cytotoxicity of both forms of ellipticine was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) cytotoxicity test.

MATERIALS AND METHODS

Chemicals and enzymes

Ellipticine, NADPH, and calf thymus DNA were from Sigma Chemical Co. (St. Louis, MO, USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described (Stiborova *et al.* 2001). Other chemicals were of analytical grade or better. 12-Hydroxy- and 13-hydroxyellipticine were isolated from multiple high performance liquid chromatogra-

phy runs of ethyl acetate extracts of incubations containing ellipticine, DNA, NADPH and rat hepatic microsomes (Indra *et al.* 2013) isolated as described (Stiborova *et al.* 2004). The poly(ethylene oxide)-block-poly(allyl glycidyl ether) (PAGE-PEO) block copolymer (P 1191 nanoparticles), prepared and characterized as described previously (Hruby *et al.* 2005), were a gift of Dr. M. Hruby (Institute of Macromolecular Chemistry AS CR, Prague, Czech Republic). Ellipticine, dissolved in dimethyl sulfoxide (DMSO) into a saturation concentration, was gradually added into a water solution of polymeric nanoparticles P119 and the mixture incubated at 23 °C for 24 h. A concentration of a non-covalently bound ellipticine in a hydrophobic core was determined spectrophotometrically (at 310 nm).

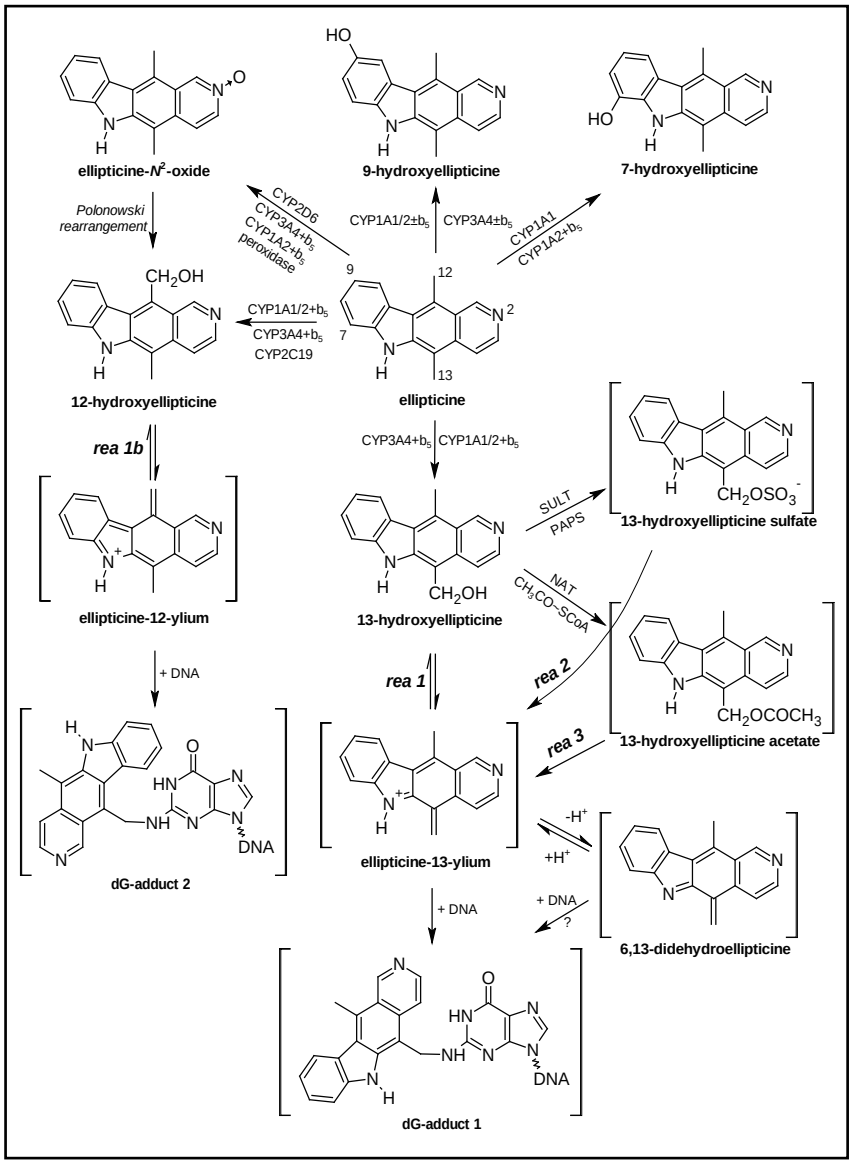

Fig. 1. Ellipticine metabolism by CYPs showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and/or are not yet structurally characterized. Reactions (rea) 1, 2 and 3 lead to formation of ellipticine-13-ylium from 13-hydroxyellipticine, 13-hydroxyellipticine sulfate and 13-hydroxyellipticine acetate, respectively, and rea 1b to formation of ellipticine 12-ylium. ? in figure indicates that the mechanisms of this reaction are not known. b_5 – cytochrome b_5; CH_3CO-SCoA – acetyl-S-coenzyme A; NAT – *N,O*-acetyltransferase; PAPS – 3′-phosphoadenosyl-5′-phosphosulfate; SULT – sulfotransferase.

Ellipticine micelles stability and its release from micelles in vitro

Stability of ellipticine-micelles and ellipticine release from these micelles were determined by a dialysis method as described in our previous work (Stiborova *et al.* 2014a) after their incubation in 0.1 mol.L^{-1} sodium phosphate buffer pH 5.0 and 7.4 at 25 °C for 0 till 95 h and measured spectrophometrically (at 310 nm). The results found in this our former study demonstrated that ellipticine as a hydrophobic base compound is easily released from the micelles into the water environment under both pH used, predominantly under the acidic conditions (Stiborova *et al.* 2014a).

Cell cultures

Human leukemia HL-60 cells (a promyelocytic line) were from the collection of cell lines of the Department of Pediatric Hematology and Oncology, 2nd Medical School, Charles University and University Hospital Motol (Prague, Czech Republic). HL-60 cells were cultivated as described previously (Poljakova *et al.* 2007). Briefly, the cells were cultivated in Iscove's modified Dulbecco's medium (IMDM, Biochrom AG, Berlin, Germany), high-glucose type, supplemented with 4 mM L-glutamine, 10% fetal calf serum (PAA Laboratories, Pasching, Austria), 100 units per ml of penicillin and 100 μg.mL^{-1} streptomycin (PAA, Vienna, Austria) and 0.3% (*w*/*v*) $NaHCO_3$ at 37 °C, 5% CO_2 and 95% atmospheric humidity.

MTT test

The cytotoxicity of free ellipticine and its micellar form 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. Ellipticine dissolved in water in the presence of acetic acid [for details, see (Stiborova *et al.* 2007b)] (5 μL) in final concentrations of 0.01–10 μmol.L^{-1} or the same concentrations of ellipticine present in ellipticine-micelles in a distilled water solution was added. Control cells and medium controls without cells received 5 μL of distilled water or micelles without ellipticine. Tumor cell viability was

evaluated by MTT test as previously described (Poljakova *et al.* 2007). Briefly, after 24 h of incubation at 37 °C in 5% CO_2 saturated atmosphere the MTT solution (2 mg.mL^{-1}) 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. 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 half maximal inhibition concentration (IC_{50}) values were calculated from the linear regression of the dose-log response curves.

Treatment of HL-60 cells with free ellipticine and its micellar form for DNA adduct analyses

HL-60 cells were seeded 24 h prior to treatment with ellipticine and its micellar form at a density of 1×10^5 cells.mL^{-1} in two 75 cm^2 culture flasks in a total volume of 20 mL of IMDM. The cells were treated with ellipticine that was dissolved in water in the presence of acetic acid [for details, see (Stiborova *et al.* 2007b)] in final concentrations of 0.01, 0.1, 1.0, 2.0 or 5.0 μmol.L^{-1} or the same concentrations of ellipticine present in ellipticine-micelles in a distilled water solution. The HL-60 cells cultivated in IMDM without ellipticine or with micelles without ellipticine were used as controls. After 24 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 from HL-60 cells by a standard phenol/chloroform extraction method as described (Poljakova *et al.* 2007).

Tab. 1. DNA adduct formation by ellipticine and ellipticine in micelles in human HL-60 cells (A) and their cytotoxicity to these cells (B). Data shown are mean ± standard error of mean of three determinations.

(A)

	Total ellipticine-derived DNA adducts[a] (RAL × 10^{-7})	
Concentrations (μmol.L^{-1})	**Ellipticine**	**Ellipticine-micelles**
0	N.D.	N.D.
0.01	N.D.	N.D.
0.1	N.D.	N.D.
1.0	1.0 ± 0.2	0.9 ± 0.2
2.0	2.5 ± 0.3	2.3 ± 0.3
5.0	10.1 ± 1.2	9.8 ± 1.0

(B)

IC_{50} (mmol.L^{-1})[b] for	
Ellipticine	Ellipticine-micelles
1.3 ± 0.3	1.4 ± 0.3

HL-60 cells were exposed to ellipticine or ellipticine in micelles for 24 h. DNA adducts were analyzed by the nuclease P1 version of the ^{32}P-postlabeling assay (Stiborova *et al.* 2001).
[a]Total DNA adduct levels (sum of adducts 1 and 2 in Figure 2) in cells exposed to 0 – 5 μmol.L^{-1} free ellipticine or ellipticine in micelles. Levels of DNA adducts are expressed as mean RAL (relative adduct labeling) and standard deviations of three determinations.
[b]IC_{50} values were calculated from the linear regression of the dose-log response curves using MTT test. Values are means and standard deviations of 8 determinations. N.D. – not detectable, RAL <10^9 adducts/normal nucleotides.

^{32}P-postlabeling of ellipticine-derived DNA adducts

DNA samples isolated from HL-60 cells treated both with elliptiicine and ellipticine-micelles were analyzed for the presence of ellipticine-derived DNA adducts by the nuclease P1 version of the ^{32}P-postlabeling method as described (Stiborova *et al.* 2001, 2003, 2004, 2011; Stiborova & Frei 2014). Samples of calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine (Stiborova *et al.* 2004, 2007a) analyzed by the same method were used to compare DNA adduct spot patterns. Chromatographic conditions for thin layer chromatography (TLC) on polyethylenimine-cellulose plates (10 cm × 20 cm; Macherey-Nagel, Düren, Germany) were: D1, 1.0 mol.L^{-1} sodium phosphate, pH 6.8; D3: 3.5 lithium-formate, 8.5 mol.L^{-1} urea, pH 3.5; D4, 0.8 mol.L^{-1} lithium chloride, 0.5 mol.L^{-1} Tris-HCl, 8.5 mol.L^{-1} urea, pH 8; D5, 1.7 mol.L^{-1} sodium phosphate, pH 6. After chromatography TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Stiborova *et al.* 2001, 2004, 2007a; Poljakova *et al.* 2007).

Statistical analyses

For statistical data analysis we used Student's *t*-test. All *p*-values are two-tailed and considered significant at the 0.05 level.

RESULTS

Ellipticine-derived DNA adduct formed by free ellipticine and ellipticine-micelles in human leukemia HL-60 cells

In order to determine the formation of ellipticine-derived DNA adducts as the biological end-point of the pharmacological and genotoxic effects of this drug in HL-60 cells exposed to increasing concentrations of free ellipticine or ellipticine in micelles (0; 0.01; 0.1; 1.0; 2.0 and 5.0 μmol.L^{-1}), the nuclease P1 version of the ^{32}P-postlabeling assay (Stiborova *et al.* 2001, 2003, 2004, 2011; Stiborova & Frei 2014) was used. Two major ellipticine-DNA adducts (spots 1 and 2 in Figure 2) generated from two ellipticine metabolites, 13-hydroxyellipticine and 12-hydroxyellipticine, respectively (Stiborova *et al.* 2004, 2007a, 2010, 2013; Moserova *et al.* 2008; Kotrbova *et al.* 2011), were formed in DNA of HL-60 cells

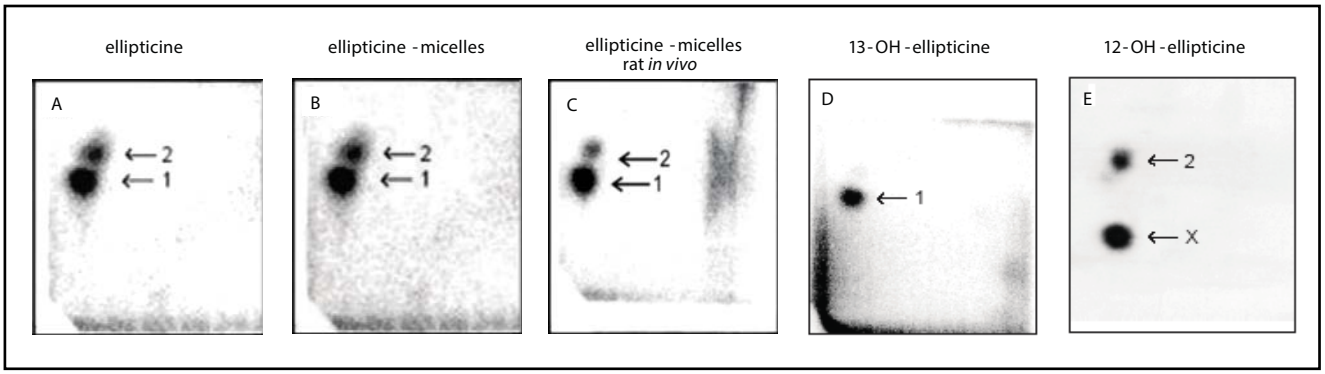

Fig. 2. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from HL-60 cell treated for 24 h with 5 μM ellipticine (A), with ellipticine in micelles (B), of liver DNA of Wistar rats treated *i.p.* with 10 mg ellipticine in micelles per kilogram body weight (C), of calf thymus DNA reacted with 13-hydroxyellipticine (D) and 12-hydroxyellipticine (E). Adduct spots 1 and 2 correspond to the DNA adducts formed by 13-hydroxyellipticine and 12-hydroxyellipticine. Besides adduct 2 formed by 12-hydroxyellipticine, another strong adduct (spot X in panel E), which was not found in any other activation systems or *in vivo* was generated. Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay.

cultivated for 24 h with 1.0; 2.0 and 5.0 μmol.L^{-1} of free ellipticine and ellipticine in micelles. The formation of these DNA adducts was dependent on concentrations of both ellipticine and ellipticine in micelles used in the experiments (Table 1A). In contrast to 1.0; 2.0 and 5.0 μmol.L^{-1} concentrations of both forms of ellipticine, no ellipticine-derived DNA adducts were found in these cells treated with their lower concentrations (0.01 and 0.1 μmol.L^{-1}). No adducts were also detectable in the control incubations, where HL-60 cells were treated without ellipticine or with micelles (P 199 nanoparticles) absenting ellipticine. The levels of adducts were quantified by determining the ^{32}P radioactivity of the adducts and expressed as relative adduct labeling (RAL).

Essentially the same levels of ellipticine-derived DNA adducts were formed in DNA of HL-60 cells exposed to free ellipticine and ellipticine in micelles (Table 1A, Figure 2). These results indicate that both free ellipticine and ellipticine present in micelles are capable of entering the HL-60 cells, where they are oxidatively activated by CYP and peroxidase enzymes (CYP1A1, cyclooxygenase-1 and myeloperoxidase) expressed in the membrane of endoplasmic reticulum (CYP1A1) and cytoplasm (peroxidases) of these cells, respectively (Poljakova *et al.* 2007), to 12-hydroxy- and 13-hydroxyellipticine that finally form the DNA adducts (Figure 1). The mechanism of the ellipticine enter into the cells seems to be the transfer of free ellipticine released from the micelles across the membrane (Stiborova *et al.* 2014a).

Cytotoxicity of free ellipticine and ellipticine in micelles on human leukemia HL-60 cells

In order to determine the cytotoxicity of free ellipticine and ellipticine in micelles on human leukemia HL-60 cells, the cells were cultivated with increasing concentrations of ellipticine and ellipticine-micelles for 24 h and viable cells were detected with MTT assay. No toxic effect of micelles without ellipticine on HL-60 cells was found (Figure 3). Cytotoxicity of free ellipticine and ellipticine in micelles on the HL-60 cells was dose-dependent (Figure 3). The IC_{50} values for free ellipticine were similar to those for ellipticine in micelles (Table 1B).

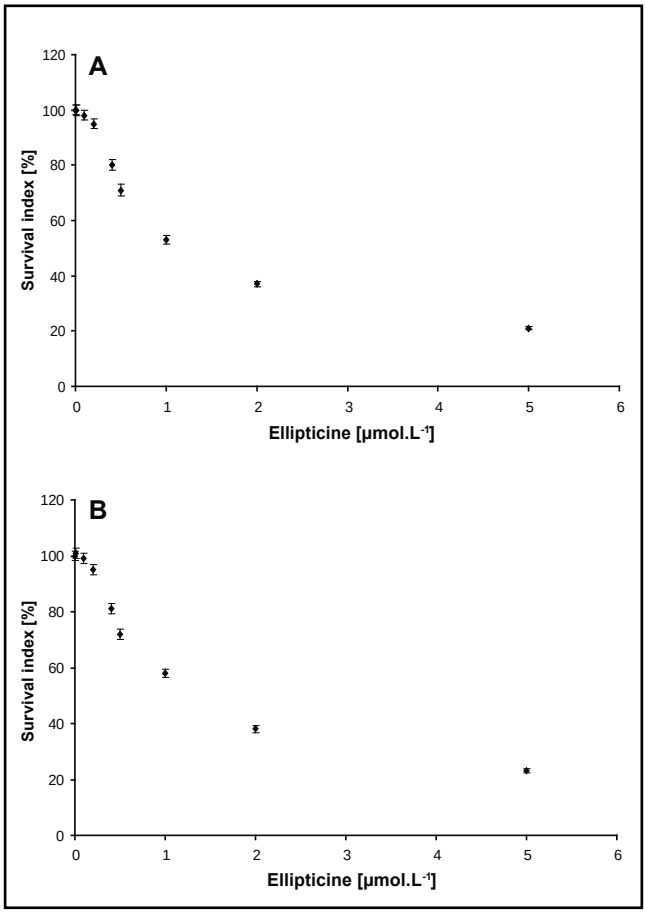

Fig. 3. Cytotoxicity of ellipticine (A) and ellipticine in micelles (B) on HL-60 cells after 24 h exposure to these forms of ellipticine. Cytotoxicity was determined by the MTT assay. Values are means and standard deviations of 8 determinations.

DISCUSSION

This study focused on comparison of toxicity of ellipticine and its micellar form [the poly(ethylene oxide)-block-poly(allyl glycidyl ether) block copolymer, P 119 nanoparticles] on human leukemia HL-60 cells *in vitro*.

The results found in the study demonstrate cytotoxicity of both free ellipticine and this drug encapsulated in micelles on the tested leukemia cells. This finding indicates that both forms of ellipticine (free ellipticine and ellipticine in micelles) enter HL-60 cells by their transport across the cell membrane and produce the toxic effects on these cells. These results are in concordance with the findings shown in our former work, where an easy transport of not only the free ellipticine, but also its micellar form, across the biological membrane and their entering the cells of various organs of rats *in vivo*, were found (Stiborova *et al.* 2014a). The novel micellar form of ellipticine was found in the *in-vivo* experiments to be well distributed in rats by their transport from peritoneum into the different rat organs, even into rat brain.

The cytotoxic effects of ellipticine and its encapsulated form on HL-60 cells were analogous; no differences in the values of IC_{50} for both forms of elllipticine were found (see Table 1B). Since formation of covalent ellipticine-derived DNA adducts is one of the most important mechanisms responsible for the ellipticine pharmacological and cytotoxic effects in many cancer cells (Stiborova *et al.* 2011, 2014b; Stiborova & Frei 2014), we also investigated whether these adducts are formed by a micellar form of ellipticine and might be responsible for its cytotoxicity on the tested leukemia cells. Cultivation of HL-60 cells with both forms of ellipticine led to formation of the covalent ellipticine-derived DNA adducts in these cells. Similar to analogous cytotoxicity of free ellipticine and ellipticine in micelles on HL-60 cells, no significant differences in levels of these adducts formed by both these forms of ellipticine were found.

In contrast to analogous effects of a free ellipticine and its micellar form in *in vitro* experiments with leukemia HL-60 cells found in the present work, a different fate of both forms of the drug was found in rats *in vivo*. The levels of ellipticine-DNA adducts formed in rat tissues after their administration with ellipticine-micelles were one order of magnitude lower in most organs than in those of rats exposed to free ellipticine, with an exception of brain, where levels of ellipticine-DNA adducts formed by ellipticine in micelles were higher than in DNA of brain of rats treated with free ellipticine. This finding emphasizes that this micellar form of ellipticine might probably be employed to treat the brain tumors, treatment of which by several cytostatics is usually limited because of strict selectivity of the hematoencephalic barrier (Stiborova *et al.* 2014a). The lower levels of ellipticine-DNA adducts in rat organs *in vivo* indicate a gradual release of ellipticine from micelles that might produce the enhanced permeation and retention effect of the ellipticine-micellar delivery system *in vivo*. These findings suggest a suitability of this ellipticine micellar form to deliver the drug to target tissues.

The results of the present study, showing the analogous effects of free ellipticine and ellipticine in micelles in the system of cancer cells *in vitro* (*i.e.* leukemia HL-60 cells), demonstrate certain limitations of utilization of such *in vitro* systems to investigate the pharmacological and cytotoxic effects of various forms of ellipticine. The similar results were also found in another study, where the cytotoxicity of several forms of nanoparticular doxorubicin on different prostatic cells was investigated (Gumulec *et al.* 2014). The biodistribution of the micellar nanotransporter of ellipticine *in vivo* (Stiborova *et al.* 2014a), and perhaps other anticancer drugs, dictates their biological effects. Therefore, in order to resolve the efficiencies of a micellar form of ellipticine on cancer cells *in vivo*, the experiments employing administration of the drug in a nanotransporter form in animal models bearing tumors (xenografts) should be performed.

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