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Effectiveness of human cytochrome P450 3A4 present in liposomal and microsomal nanoparticles in formation of covalent DNA adducts by ellipticine

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Abstract

OBJECTIVES: Ellipticine is an anticancer agent that functions through multiple mechanisms participating in cell cycle arrest and initiation of apoptosis. This drug forms covalent DNA adducts after its enzymatic activation with cytochrome P450 (CYP), which is one of the most important ellipticine DNA-damaging mechanisms of its cytotoxic effects. The improvements of cancer treatment are the major challenge in oncology research. Nanotransporters (nanoparticles) are promising approaches to target tumor cells, frequently leading to improve drug therapeutic index. Ellipticine has already been prepared in nanoparticle forms. However, since its anticancer efficiency depends on the CYP3A4-mediated metabolism in cancer cells, the aim of our research is to develop nanoparticles containing this enzyme that can be transported to tumor cells, thereby potentiating ellipticine cytotoxicity.
METHODS: The CYP3A4 enzyme encapsulated into two nanoparticle forms, liposomes and microsomes, was tested to activate ellipticine to its reactive species forming covalent DNA adducts. Ellipticine-derived DNA adducts were determined by the ^{32}P-postlabeling method.
RESULTS: The CYP3A4 enzyme both in the liposome and microsome nanoparticle forms was efficient to activate ellipticine to species forming DNA adducts. Two DNA adducts, which are formed from ellipticine metabolites 12-hydroxy- and 13-hydroxyellipticine generated by its oxidation by CYP3A4, were formed by both CYP3A4 nanoparticle systems. A higher effectiveness of CYP3A4 in microsomal than in liposomal nanoparticles to form ellipticine-DNA adducts was found.
CONCLUSION: Further testing in a suitable cancer cell model is encouraged to investigate whether the DNA-damaging effects of ellipticine after its activation by CYP3A4 nanoparticle forms are appropriate for active targeting of this enzyme to specific cancer cells.

ORIGINAL ARTICLE

Abbreviations:
CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CYP - cytochrome P450
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
POR - NADPH:CYP oxidoreductase
NAT - *N,O*-acetyltransferase
RAL - relative adduct labeling
SULT - sulfotransferase

INTRODUCTION

Cancer treatment is one of the most difficult problems in clinic practice. Many approaches were utilized to develop novel antitumor drugs, which could be applied with other therapeutic modalities. One of the groups of drugs that exhibit the high antitumor effectiveness is the group of DNA-damaging drugs. Nevertheless, their application is often limited by their side effects [for a summary see (Stiborova *et al.* 2011; Kizek *et al.* 2012; Stiborova & Frei, 2014)]. Therefore, the aim of many laboratories developing novel anticancer drugs, is design of drugs with less side effects. One of the possibilities is the usage of nanoparticles (nanotransporters), the drug forms that often exhibit lower side effects, and moreover even higher antitumor effects than their parental free drugs. The advantages of the drug delivery performed by nanocarriers such as iron oxides, gold, biodegradable polymers, dendrimers, and lipid based carriers (*i.e.* liposomes or micelles) have been extensively investigated [for a summary see (Wu *et al.* 2012; Masood *et al.* 2013; Heger *et al.* 2014)]. The DNA-damaging drugs such as doxorubicin, etoposide and ellipticine encapsulated into nanoparticles have been already prepared in our laboratories and their cytotoxicity on several cancer cells has been investigated (Blazkova *et al.* 2013; Gumulec *et al.* 2014; Heger *et al.* 2014; Stiborova *et al.* 2014a; 2015b; Dostalova *et al.* 2016). However, because anticancer efficiency of some of the above mentioned drugs (*i.e.,* doxorubicin and ellipticine) depends on their metabolism in cancer cells, another aim of our research is to develop nanocarriers that will contain not only these drugs, but also the enzymatic systems, namely, the nanocarriers that can transport these enzymes to tumor cells where they can potentiate the drug antitumor effects.

Ellipticine (Figure 1) is efficient anticancer compound that functions through multiple mechanisms participating in cell cycle arrest and initiation of apoptosis [for a summary see (Stiborova *et al.* 2001; 2006; 2011; 2015a; Garbett & Graves, 2004; Kizek *et al.* 2012; Stiborova and Frei, 2014)]. The predominant mechanisms of ellipticine's biological effects were suggested to be (i) intercalation into DNA (Garbett and Graves, 2004; Tmejova *et al.* 2014) and (ii) inhibition of topoisomerase II (Garbett & Graves, 2004; Stiborova *et al.* 2011; Kizek *et al.* 2012; Stiborova & Frei, 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; 2003; 2004; 2007a; 2007b; 2008; 2011; 2012a; 2012b; 2015b; Kizek *et al.* 2012; Stiborova & Frei, 2014; Kotrbova *et al.* 2011), suggesting an additional DNA-damaging effect of ellipticine. Of the CYP enzymes investigated, human CYP3A4 followed by CYP1A1 and 1B1 are the most active enzymes oxidizing ellipticine to 12-hydroxy- and 13-hydroxyellipticine, the reactive metabolites that dissociate to ellipticine-12-ylium and ellipticine-13-ylium, which bind to DNA (Figures 1 and 2) (Stiborova *et al.* 2004; 2007a; 2008; 2011; 2015a). The CYP1A isoforms also efficiently form the other ellipticine metabolites, 7-hydroxy- and 9-hydroxyellipticine, which are the detoxification products (Figure 1).

The ellipticine-derived DNA adducts that were found in *in-vitro* incubations of ellipticine with DNA and enzymes activating this drug, were generated also in several cancer cell lines (Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007; 2009; 2011; Martinkova *et al.* 2009; Stiborova *et al.* 2011; Stiborova and Frei, 2014) and in rat mammary adenocarcinoma *in vivo* (Stiborova *et al.* 2011) (Fig. 2). This DNA-damaging effect has been considered as one of the major mechanisms responsible for ellipticine high cytotoxic effects on cancer cells (Stiborova *et al.* 2003; 2007b; 2008; 2011; 2014b; 2015a; Stiborova & Frei, 2014).

In the present study, we aimed to prepare one of the enzymes, which oxidize the studied drug ellipticine to metabolites increasing its anticancer efficiency, in its nanoparticle forms. The CYP3A4 enzyme was chosen for this study. Because the CYP enzymes are the proteins naturally located in a membrane of the endoplasmic reticulum of cells dictating their enzymatic activity (Guengerich, 2008; 2011), the lipid based nanocarriers should be the suitable systems for these enzymes (Stiborova *et al.* 2001; Kotrbova *et al.* 2011). Therefore, the CYP3A4 in the liposomal nanoparticles was prepared and used for evaluation of its catalysis to activate ellipticine. Liposomes are artificially prepared, self-assembled structures composed of phospholipids in which an outer lipid bilayer surrounds a central aqueous space. It should be mentioned that several CYPs of animal models such as rats and rabbits reconstituted with NADPH:CYP reductase (POR) in liposomal vesicles were efficient to activate ellipticine, but such activity of human CYP3A4 in this artificial system has not been tested as yet (Kotrbová *et al.* 2006). Moreover, no comparison of effectiveness of the CYP3A4-liposome system with that of the natural nanoparticle membrane system, microsomes, was investigated. Therefore, such comparison is another target of this work.

MATERIALS AND METHODS

Chemicals and material

Ellipticine, dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, dilauroyl phosphatidylserine,

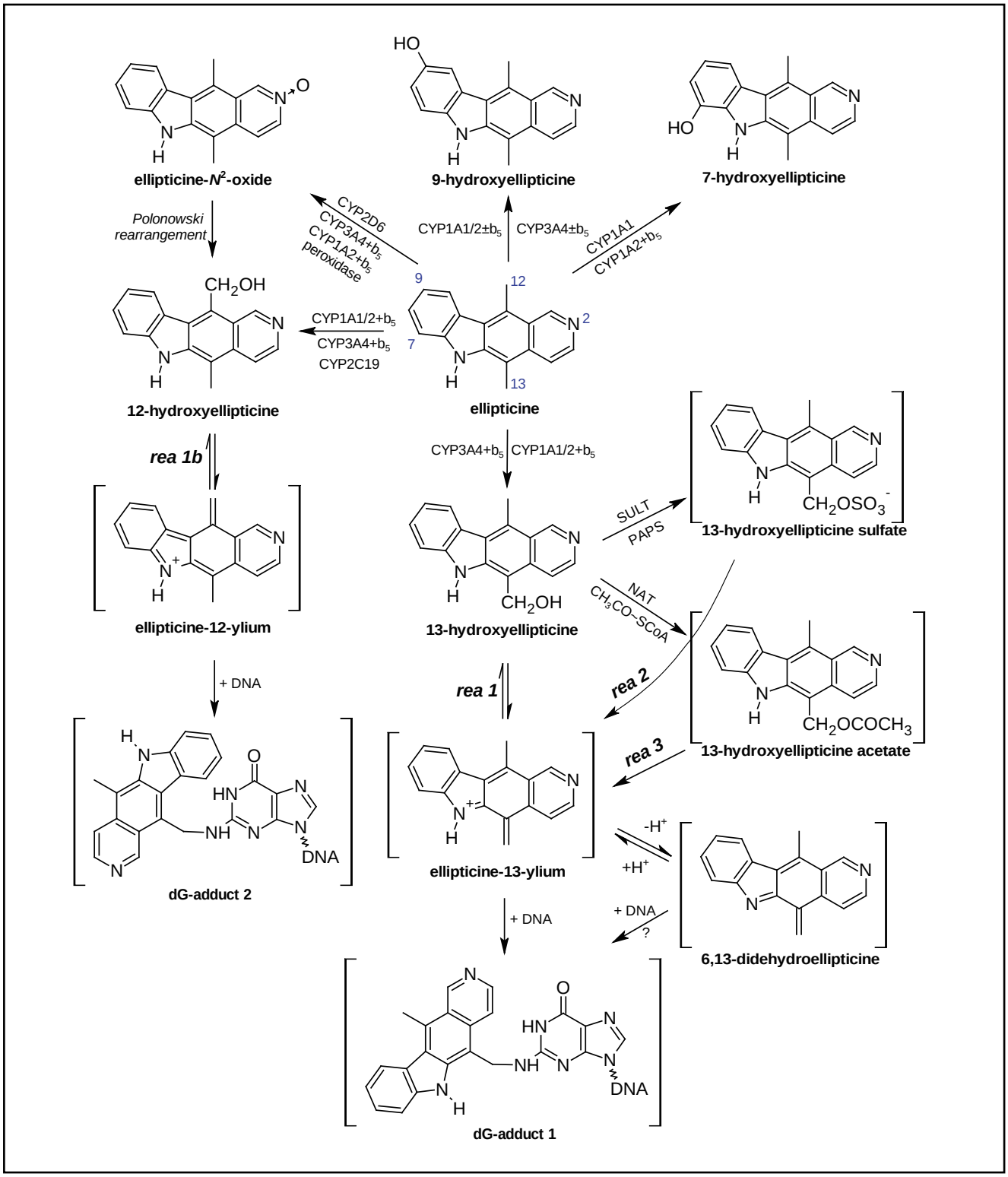

Fig. 1. Scheme of ellipticine metabolism catalyzed by CYPs showing the identified metabolites and those proposed to form DNA adducts. The compounds showed in brackets were not detected under the experimental conditions and/or not yet structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in our previous studies (Stiborova *et al.* 2004; 2008; 2012a; Kotrbova *et al.* 2011). Rea 1, 2 and 3 are reactions leading to ellipticine-13-ylium from 13- hydroxyellipticine, 13-hydroxyellipticine sulfate and 13-hydroxyellipticine acetate, respectively [adopted from (Stiborova *et al.* 2004; 2012a).

chloroform, glutathione, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), NADPH, calf thymus DNA, and others were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity (purity meets the standards of

American Chemical Society), unless noted otherwise. Human recombinant CYP3A4 was a gift of Professor Pavel Anzenbacher (Palacky University at Olomouc, Czech Republic). Rabbit liver NADPH:CYP reductase was purified from rabbit liver microsomes as described (Yasukochi *et al.* 1979). Both these pure enzymes were incorporated into liposomes and used in further experiments as shown below (Chapter – *Determination of DNA adduct formation by ellipticine in vitro by ^{32}P-postlabeling*). Supersomes™ are microsomes isolated from insect cells transfected with a baculovirus construct containing cDNA of human CYP3A4, and which also express POR, were purchased from Gentest Corp. (Woburn, MI, USA).

Preparation of liposomes encapsulating CYP3A4 and POR

Liposomes were prepared as described previously (Stiborova *et al.* 2001) with small modification. Briefly, liposomes were prepared from dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine and dilauroyl phosphatidylserine. Each individual lipid was dissolved in chloroform (20 mg/ml) and mixed in a ratio of 1:1:1. A lipid film was obtained by rotary evaporation of chloroform. Residual chloroform was removed by a stream of nitrogen. The lipid film was further dispersed with 50 mM HEPES/KOH buffer, pH 7.4, containing 3 mM reduced glutathione, 0.1 mM CHAPS and ultrasonicated twice at 20 °C for 3 min (6 min together). The appropriate amounts of human CYP3A4 and rabbit POR (50–250 pmol in a ratio of 1:1) were added to the prepared dispersion and incubated at 20 °C for 10 min.

Determination of DNA adduct formation by ellipticine in vitro by ^{32}P-postlabeling

Incubation mixtures used to assess DNA adduct formation by ellipticine (Stiborova *et al.* 2001; 2004; 2012a; 2015a; Kotrbova *et al.* 2011) activated with liposomal and Supersomal nanoparticles containing CYP3A4 and POR in a ratio of 1:1 consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 10–250 pmol CYP3A4 with the same amounts of POR in nanoparticles, 0.1 mM ellipticine (dissolved in 7.5 µl dimethyl sulfoxide), and 0.5 mg of calf thymus DNA in a final volume of 750 µl. The reaction was initiated by adding 0.1 mM ellipticine. Incubations at 37 °C were carried out for 60 min. Ellipticine-derived DNA adduct formation has been shown to be linear up to 90 min (Stiborova *et al.* 2001). Control incubations were carried out either without enzymatic system, without NADPH, without DNA, or without ellipticine. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method.

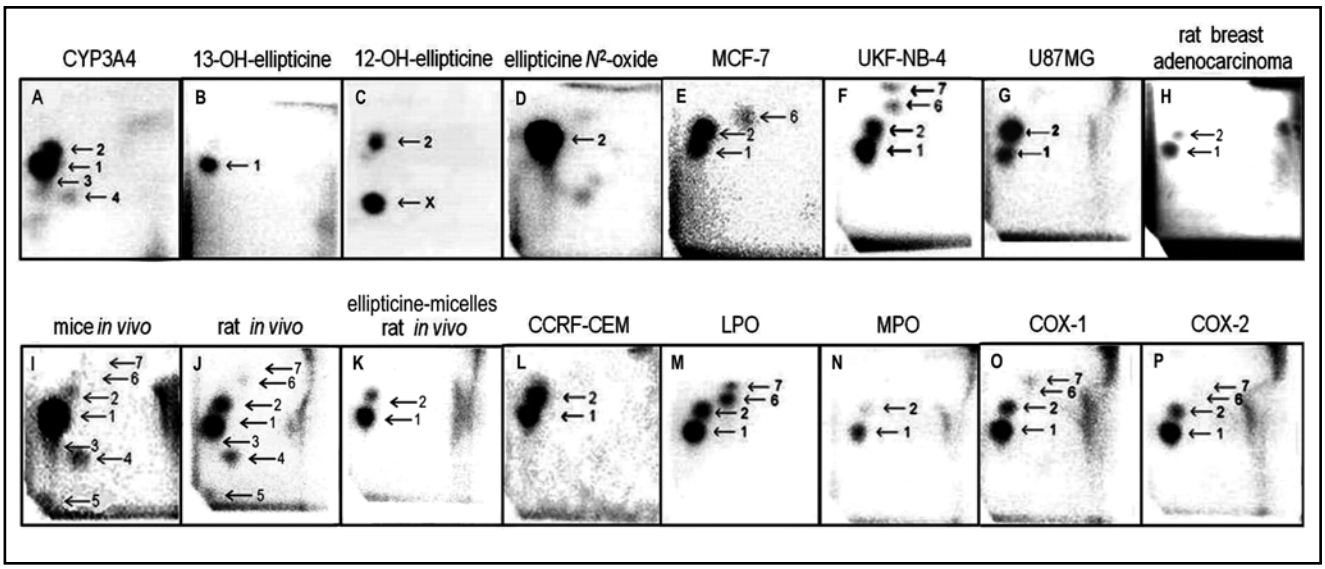

Fig. 2. Autoradiographic profiles of ellipticine-derived DNA adducts analyzed with the ^{32}P-postlabeling assay. Adduct profiles obtained from calf thymus DNA reacted with ellipticine and CYP3A4 in Supersomes™ (Stiborova *et al.* 2004) (A), from calf thymus DNA reacted with 13-hydroxyellipticine (Stiborova *et al.* 2004) (B), 12-hydroxyelipticine (Stiborova *et al.* 2007a) (C), ellipticine N^2-oxide (Stiborova *et al.* 2004) (D), from DNA of breast adenocarcinoma MCF-7 cells (Borek-Dohalska *et al.* 2004) (E), neuroblastoma UK-NB-4 cells (Poljakova *et al.* 2009) (F) and glioblastoma U87MG cells (Martinkova *et al.* 2009) (G) exposed to 10 µM ellipticine, from DNA of breast adenocarcinoma of Wistar rats treated *i.p.* with 4 mg ellipticine per kilogram body weight (Stiborova *et al.* 2011) (H), from liver DNA of C57BL/6 mice treated *i.p.* with 10 mg ellipticine per kilogram body weight (Stiborova *et al.* 2008) (I), from liver DNA of Wistar rats treated *i.p.* with 10 mg ellipticine per kilogram body weight (Stiborova *et al.* 2014a) (J), from liver DNA of Wistar rats treated *i.p.* with 10 mg ellipticine in micelles per kilogram body weight (Stiborova *et al.* 2014a) (K) and CCRF-CEM cells (Poljaková *et al.* 2007) (L) treated with 10 µM ellipticine, from calf thymus DNA reacted with ellipticine and bovine lactoperoxidase (LPO) (Stiborova *et al.* 2007a) (M), human myeloperoxidase (MPO) (Stiborova *et al.* 2007a) (N), ovine cyclooxygenase (Stiborova *et al.* 2007a) (COX)-1 (O) and human COX-2 (Stiborova *et al.* 2007a) (P). Adopted from (Stiborova *et al.* 2014a). Adduct spots 1-7 correspond to the ellipticine-derived DNA adducts. Besides adduct 2 formed by 12-hydroxyellipticine (C), another strong adduct (spot X in panel C), which was not found in any other activation systems or *in vivo* was generated.

DNA adducts were analyzed with the nuclease P1 version of the ^{32}P-postlabeling technique (Reddy & Randerath, 1986; Indra *et al.* 2014), which was found to be suitable to detect and quantify the ellipticine-derived DNA adducts (Stiborova *et al.* 2001). Resolution of the adducts by thin-layer chromatography using polyethylenimine-cellulose plates (Macherey and Nagel, Düren, Germany) was carried out as reported (Stiborova *et al.* 2001; Aimova *et al.* 2007). DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Schmeiser *et al.* 2013).

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 AND DISCUSSION

The CYP3A4 enzyme systems in nanoparticle forms

In order to evaluate enzymatic activity of human CYP3A4 in nanoparticle forms, (*i*) CYP3A4 introduced with its reductase, POR, into liposomes and (*ii*) CYP3A4 present in a natural nanoparticle form, microsomes, were utilized.

Liposomes with CYP3A4 and POR were prepared as described in the Material and methods section and used in further experiments. The aliquots of the liposomes containing increasing amounts of CYP3A4 were added into the incubation mixtures to reach its final amounts of 50–250 pmol and these mixtures were used for activation of ellipticine to species forming ellipticine-derived DNA adducts. The details are described in the Material and methods section.

The second nanoparticle system used in the experiments were Supersomes™ (Gentest Corp., Woburn, MI, USA), microsomes isolated from insect cells transfected with a baculovirus construct containing cDNA of human CYP3A4 and its reductase, POR. Besides overexpressed human CYP3A4 and POR, these microsomes contained a low amount of other enzymes of the monooxygenase system that were present in the membrane of the insect endoplasmic reticulum (*i.e.,* microsomal epoxide hydrolase, cytochrome b_5 and its reductase, NADH:cytochrome b_5 reductase). But their levels were, in comparison to overexpresssed CYP3A4 and POR, negligible. The utilization of Supersomes™ for analysis of their efficiencies to activate ellipticine to species forming ellipticine-derived DNA adducts was analogous to that of the liposomal systems (see above).

Formation of ellipticine-derived DNA adducts by CYP3A4 present in liposomes and Supersomes™

Both nanoparticle systems (containing CYP3A4 and POR) incubated in the presence of a cofactor of POR, NADPH, and DNA activated ellipticine to metabolites forming DNA adducts (see adducts formed by CYP3A4 with POR in the liposomal form shown in Figure 2). Using the nuclease P1 version of the ^{32}P-postlabeling assay (Reddy and Randerath, 1986) found to be suitable to detect and quantify the ellipticine-derived DNA adducts (Stiborova *et al.* 2001), two adducts formed by activated ellipticine with DNA added to the incubation mixtures were detected; one major generated from ellipticine-13-ylium formed by decomposition of 13-hydroxyellipticine (Stiborova *et al.* 2004) (see adduct spot 1 in Figures 2 and 3) and one minor generated from ellipticine-12-ylium formed from 12-hydroxyellipticine (Stiborova *et al.* 2007a) (see adduct spot 2 in Figures 2 and 3). These two adducts were analogous to those formed in several and cancer cells *in vitro* and in healthy and tumour cells *in vivo* (Figure 2). An analogous pattern of ellipticine-derived DNA adducts was formed by CYP3A4 present in the second nanoparticle system tested in this work, Supersomes™. Control incubations carried out either without ellipticine, or

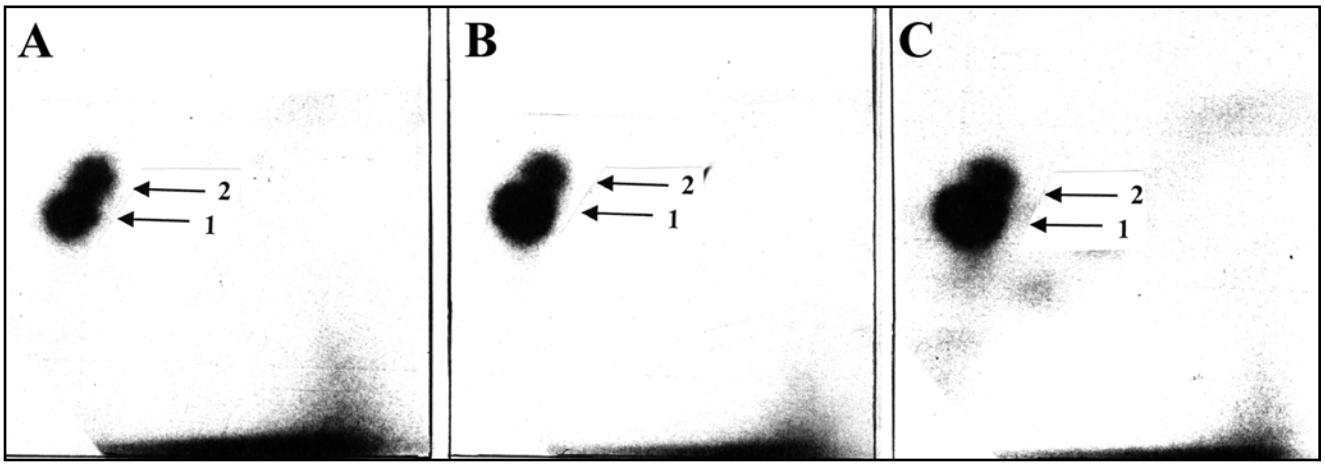

Fig. 3. Pattern of ellipticine-DNA adducts and their levels in relation to concentration of human CYP3A4 in the liposomal nanoparticles with this CYP and POR. 50 pmol (**A**), 100 pmol (**B**) and 250 pmol CYP3A4 (**C**). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right). Arrows 1 and 2 indicate the dG-adducts 1 and 2 formed from ellipticine-13-ylium and ellipticine-12-ylium, respectively (see Figures 1 and 2).

Tab. 1. Levels of ellipticine-derived DNA adducts formed from ellipticine after activation with human CYP3A4 present in liposomal and Supersomal nanoparticles. Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay.

CYP3A4 in nanoparticle	RAL[a] (mean±SD/10^7 nucleotides)[b] in dG-adduct	
CYP3A4 in liposomes	1[c]	2
No CYP3A4	Not detectable	0.20±0.03
10 pmol	Not measured	
50 pmol	1.42±0.08	0.21±0.03
100 pmol	2.31±0.15	0.20±0.03
200 pmol	4.22±0.31	0.21±0.03
250 pmol	4.53±0.34	0.20±0.03
CYP3A4 in Supersomes™		
No CYP3A4	Not detectable	0.21±0.03
10 pmol	0.75±0.05	0.21±0.03
50 pmol	2.10±0.12*	0.21±0.03
100 pmol	3.82±0.26*	0.20±0.03
200 pmol	5.21±0.33*	0.21±0.03
250 pmol	5.60±0.32*	0.21±0.03

[a]Relative adduct labeling; [b] Averages of three determinations in separate experiments; [c]see Figures 1 and 2.
Experimental conditions are described in the Materials and methods section with 10-250 pmol CYP3A4 in liposome and Supersome™ nanoparticles and 100 μM ellipticine. Comparison was performed by Student *t*-test analysis; *p<0.001, different from ellipticine activation mediated by the liposomal nanoparticles.

without DNA were free of either adduct spot even after prolonged exposure times (data not shown). Control incubations performed without the enzyme system but with ellipticine were free of adduct spot 1, but adduct spot 2 was always detected. This finding indicates that this adduct might be formed also by autooxidation (Stiborova *et al.* 2001; 2011).

As shown in Figure 3, the formation of adduct 1 seems to be dependent on concentrations of human CYP3A4 in the liposomal system present in the incubation mixture. In order to confirm this suggestion, we quantified the amounts of this adduct formed in the incubations containing CYP3A4 in both nanoparticle forms, NADPH and DNA. The levels of ellipticine-derived DNA adduct 1, whose formation is mediated by CYP3A4 that catalyze its oxidation to 13-hydroxyellipticine, are indeed dependent on amounts of CYP3A4 in both used nanoparticles, being increased with elevated amounts of CYP3A4 in the system (Table 1). The levels of DNA adduct 1 formed in the liposomal and Supersomal systems are of the same order. Nevertheless, the effectiveness of CYP3A4 present in the Supersomal system is higher than that of CYP3A4 in the liposomal nanoparticles (Table 1). Now, we can only speculate to explain this phenomenon. One of the reasons might be the fact that artificially prepared liposomes do not contain all components present in the natural system of the microsomal (Supersomal) particles that can influence the CYP3A4 enzyme activity (*i.e.,* the absence of all spectrum of membrane-making lipids, the absence of proteins of microsomes including those that are components of the monooxygenase system such as cytochrome b_5). The latter suggestion is supported by our recent results, where we found that cytochrome b_5 increases the potency of CYP3A4 to oxidize ellipticine to 13-hydroxyellipticine, thereby increasing the levels of ellipticine-derived DNA adduct 1 (Stiborova *et al.* 2012a).

CONCLUSIONS

The results of the present study demonstrate that human CYP3A4 enzyme present with its reductase, POR, in liposomal nanoparticles was enzymatically effective, namely, was capable of activating an anticancer drug ellipticine to metabolites forming DNA adducts. The efficiency of this liposomal system was similar to that mediated by the natural nanoparticle system generated from the broken endoplasmic reticulum (microsomes), where CYP3A4 is located in the cells. But rather higher levels of ellipticine-derived DNA adducts were generated by a natural nanoparticle system of Supersomal microsomes. Nevertheless, the artificially prepared liposome nanoparticles with CYP3A4 catalyzed activation of ellipticine with efficiency similar to that of the Supersomal system. Hence, they seem to be suitable for delivery of the CYP3A4 enzyme to the cancer cells. We suppose that liposomes with CYP3A4 will be applied to patients intravenously, similarly like several liposomal forms of drugs. Such application has been shown to be without a risk to patients (Vieira and Gamarra, 2016). However, for active targeting of the CYP3A4-liposome nanoparticles to specific cancer cells, their modifications with suitable ligands interacting with components of surface of the tumor cells such as antibodies or their fragments, aptamers, or small molecules including peptides, growth factors, carbohydrates, and receptor ligands (Pan & Lee, 2004; Dawidczyk *et al.* 2014; Cao *et al.* 2015; Ediriwickrema & Saltzman, 2015; Shan *et al.* 2015) should be carried out. Such CYP3A4-liposome modifications are therefore the challenge of our future research.

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REFERENCES

1 Aimova D, Svobodova L, Kotrbova V, Mrazova B, Hodek P, Hudecek J, Vaclavikova R, Frei E and Stiborova M (2007). The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism. Drug Metab Dispos **35**: 1926–1934.

2 Blazkova I, Nguyen HV, Dostalova S, Kopel P, Stanisavljevic M, Vaculovicova M, Stiborova M, Eckschlager T, Kizek R and Adam V (2013). Apoferritin modified magnetic particles as doxorubicin carriers for anticancer drug delivery. Int J Mol Sci **14**: 13391–13402.

3 Borek-Dohalska L, Frei E and Stiborova M (2004). DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. Collect Czech Chem Commun **69**: 603–615.

4 Cao Y, Zhou Y, Zhuang Q, Cui L, Xu X, Xu R and He X (2015). Antitumor effect of RGD modified PTX loaded liposome on prostatic cancer. Int J Clin Exp Med **8:** 12182–12191.

5 Dawidczyk CM, Russell LM and Searson PC (2014). Nanomedicines for cancer therapy: state-of-the-art and limitations to preclinical studies that hinder future developments. Front Chem **2***:* 1–13.

6 Dostalova S, Cerna T, Hynek D, Koudelkova Z, Vaculovic T, Kopel P, Hrabeta J, Heger Z, Vaculovicova M, Eckschlager T, Stiborova M and Adam V (2016). Site-directed conjugation of antibodies to apoferritin nanocarrier for targeted drug delivery to prostate cancer cells. ACS Applied Materials & Interfaces. **8**: 14430–1441.

7 Ediriwickrema A and Saltzman WM (2015). Nanotherapy for cancer: targeting and multifunctionality in the future of cancer therapies. ACS Biomater Sci Eng **1**: 64–78.

8 Garbett NC and Graves DE (2004). Extending nature's leads: the anticancer agent ellipticine. Curr Med Chem Anti-Cancer Agents **4**: 149–172.

9 Guengerich FP (2001). Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol **14**: 611–650.

10 Guengerich FP (2008). Cytochrome P450 and chemical toxicology. Chem Res Toxicol **21**: 70–83.

11 Gumulec J, Fojtu M, Raudenska M, Sztalmachova M, Skotakova A, Vlachova J, Skalickova S, Nejdl L, Kopel P, Knopfova L, Adam V, Kizek R, Stiborova M. Babula P and Masarik M (2014). Modulation of induced cytotoxicity of doxorubicin by using apoferritin and liposomal cages. Int J Mol Sci **15**: 22960–22977.

12 Heger Z, Skalickova S, Zitka O, Adam V and Kizek R (2014). Apoferritin applications in nanomedicine. Nanomedicine (Lond) **9**: 2233–2245.

13 Indra R, Moserova M, Kroftova N, Sulc M, Martinkova M, Adam V, Eckschlager T, Kizek R, Arlt VM and Stiborova M (2014). Modulation of human cytochrome P450 1A1-mediated oxidation of benzo[*a*]pyrene by NADPH:cytochrome P450 oxidoreductase and cytochrome b_5. Neuro Endocrinol Lett **35** (Suppl 2): 105–113.

14 Kataoka K, Matsumoto T, Yokoyama M, Okano T, Sakurai Y, Fukushima S, Okamoto K and Kwon GS (2000). Doxorubicin-loaded poly(ethylene glycol)-poly(beta-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. J Control Release **64**: 143–153.

15 Kizek R, Adam V, Hrabeta J, Eckschlager T, Smutny S, Burda JV, Frei E and Stiborova M (2012). Anthracyclines and ellipticines as DNA-damaging anticancer drugs: recent advances. Pharmacol Ther **133**: 26–39.

16 Kotrbova V, Aimova D, Brezinova A, Janouchova K, Poljakova J, Hodek P, Frei E and Stiborova M (2006). Cytochromes P450 reconstituted with NADPH:P450 reductase mimic the activating and detoxicating metabolism of the anticancer drug ellipticine in microsomes. Neuro Endocrinol Lett **27** (Suppl. 2): 18–20.

17 Kotrbova V, Mrazova B, Moserova M, Martinek V, Hodek P, Hudecek J, Frei E and Stiborova M (2011). Cytochrome b_5 shifts oxidation of the anticancer drug ellipticine by cytochromes P450 1A1 and 1A2 from its detoxication to activation, thereby modulating its pharmacological efficacy. Biochem Pharmacol **82**: 669–680.

18 Masood F, Chen P, Yasin T, Fatima N, Hasan F and Hameed A. (2013). Encapsulation of ellipticine in poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) based nanoparticles and its *in vitro* application. Mater Sci Eng C Mater Biol. Appl **33**: 1054–1060.

19 Martinkova E, Dontenwill M, Frei E and Stiborova M (2009). Cytotoxicity of and DNA adduct formation by ellipticine in human U87MG glioblastoma cancer cells. Neuro Endocrinol Lett **30** (Suppl. 1): 60–66.

20 Moserova M, Kotrbova V, Rupertova M, Naiman K, Hudecek J, Hodek P, Frei E and Stiborova M (2008). Isolation and partial characterization of the adduct formed by 13-hydroxyellipticine with deoxyguanosine in DNA. Neuro Endocrinol. Lett **29**: 728–732.

21 Pan X and Lee RJ (2004). Tumour-selective drug delivery via folate receptor-targeted liposomes. Expert Opin Drug Deliv **1**: 7–17.

22 Poljakova J, Frei E, Gomez JE, Aimova D, Eckschlager T, Hrabeta J and Stiborova M (2007). DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. Cancer Lett **252**: 270–279.

23 Poljakova J, Eckschlager T, Hrabeta J, Hrebackova J, Smutny S, Frei E, Martínek V, Kizek R and Stiborova M (2009). The mechanism of cytotoxicity and DNA adduct formation by the anticancer drug ellipticine in human neuroblastoma cells. Biochem Pharmacol **77**: 1466–1479.

24 Poljakova J, Hrebackova J, Dvorakova M, Moserova M, Eckschlager T, Hrabeta J, Göttlicherova M, Kopejtkova B, Frei E, Kizek R and Stiborova M (2011). Anticancer agent ellipticine combined with histone deacetylase inhibitors, valproic acid and trichostatin A, is an effective DNA damage strategy in human neuroblastoma. Neuro Endocrinol Lett **32** (Suppl 1): 101–116.

25 Reddy MV and Randerath K (1986). Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis **7**: 1543–1551.

26 Schmeiser HH, Stiborova M and Arlt VM (2013). ^{32}P-postlabeling analysis of DNA adducts. Methods Mol Biol **1044**: 389–401.

27 Shan L, Liu M, Wu C, Zhao L, Li S, Xu L, Cao W, Gao G and Gu Y (2015). Multi-small molecule conjugations as new targeted delivery carriers for tumor therapy. Int J Nanomedicine **10**: 5571–5591.

28 Stiborova M, Bieler CA, Wiessler M and Frei E (2001). The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. Biochem Pharmacol **62**: 1675–1684.

29 Stiborova M, Breuer A, Aimova D, Stiborova-Rupertova M, Wiessler M and Frei E (2003). DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. Int J Cancer **107**: 885–890.

30 Stiborova M, Sejbal J, Borek-Dohalska L, Aimova D, Poljakova J, Forsterova K, Rupertova M, Wiesner J, Hudecek J, Wiessler M and Frei E (2004). The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine N^2-oxide. Cancer Res **64**: 8374–8380.

31 Stiborova M, Rupertova M, Schmeiser HH and Frei E (2006). Molecular mechanisms of antineoplastic action of an anticancer drug ellipticine. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **150**: 13–23.

32 Stiborova M, Poljakova J, Ryslava H, Dracinsky M, Eckschlager T and Frei E (2007a). Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. Int J Cancer **120**: 243–251.

33 Stiborova M, Rupertova M, Aimova D, Ryslava H and Frei E (2007b). Formation and persistence of DNA adducts of anticancer drug ellipticine in rats. Toxicology **236**: 50–60.

34 Stiborova M, Arlt VM, Henderson CJ, Wolf CR, Kotrbova V, Moserova M, Hudecek J, Phillips DH and Frei E (2008). Role of hepatic cytochromes P450 in bioactivation of the anticancer drug ellipticine: studies with the hepatic NADPH:cytochrome P450 reductase null mouse. Toxicol Appl Pharmacol **226**: 318–327.

35 Stiborova M, Moserova M, Mrazova B, Kotrbova V and Frei E (2010). Role of cytochromes P450 and peroxidases in metabolism of the anticancer drug ellipticine: additional evidence of their contribution to ellipticine activation in rat liver, lung and kidney. Neuro Endocrinol Lett **31** (Suppl. 2): 26–35.
36 Stiborova M, Rupertova M and Frei E (2011). Cytochrome P450- and peroxidase-mediated oxidation of anticancer alkaloid ellipticine dictates its anti-tumor efficiency. Biochim Biophys Acta **1814**: 175–185.
37 Stiborova M, Indra R, Moserova M, Cerna V, Rupertova M, Martínek V, Eckschlager T, Kizek R and Frei E (2012a). Cytochrome b_5 increases cytochrome P450 3A4-mediated activation of anticancer drug ellipticine to 13-hydroxyellipticine whose covalent binding to DNA is elevated by sulfotransferases and *N,O*-acetyltransferases. Chem Res Toxicol **25**: 1075–1085.
38 Stiborova M, Poljakova J, Martínkova E, Ulrichova J, Simanek V, Dvorak Z and Frei E (2012b). Ellipticine oxidation and DNA adduct formation in human hepatocytes is catalyzed by human cytochromes P450 and enhanced by cytochrome b_5. Toxicology **302**: 233–241.
39 Stiborova M and Frei E (2014). Ellipticines as DNA-targeted chemotherapeutics. Current Med Chem **21**: 575–591.
40 Stiborova M, Manhartova Z, Hodek P, Adam V, Kizek R and Frei E (2014a). Formation of DNA adducts by ellipticine and its micellar form in rats – a comparative study. Sensors **14**: 22982–22997.
41 Stiborova M, Poljakova J, Mrizova I, Borek-Dohalska L, Eckschlager T, Adam V, Kizek R and Frei E (2014b). Expression levels of enzymes metabolizing an anticancer drug ellipticine determined by electromigration assays influence its cytotoxicity to cancer cells – a comparative study. Int J Electrochem Sci **9**: 5675–5689.
42 Stiborova M, Cerna V, Moserova M, Mrizova I, Arlt VM and Frei E (2015a). The anticancer drug ellipticine activated with cytochrome P450 mediates DNA damage determining its pharmacological efficiencies: studies with rats, hepatic cytochrome P450 reductase null (HRN™) mice and pure enzymes. Int J Mol Sci **16**: 284–306.
43 Stiborova M, Manhartova Z, Hodek P, Adam V, Kizek R, Eckschlager T and Frei E (2015b). Cytotoxicity of and DNA adduct formation by ellipticine and its micellar form in human leukemia cells *in vitro*. Neuro Endocrinol Lett **36** (Suppl. 1): 22–28.
44 Tmejova K, Krejcova L, Hynek D, Adam V, Babula P, Trnkova L, Stiborova M, Eckschlager T and Kizek R (2014). Electrochemical study of ellipticine interaction with single and double stranded oligonucleotides. Anti-Cancer Agent Med **14**: 331–340.
45 Vieira DB, Gamarra LF (2016). Getting into the brain: liposome-based strategies for effective drug delivery across the blood-brain barrier. Int J Nanomedicine **11**: 5381–5414.
46 Wu Y, Sadatmousavi P, Wang R, Lu S, Yuan YF and Chen P (2012). Self-assembling peptide-based nanoparticles enhance anticancer effect of ellipticine *in vitro* and *in vivo. Int* J Nanomedicine **7**: 3221–3233.
47 Yasukochi Y, Peterson JA ad Masters B (1979). NADPH-cytochrome c (P450) reductase: spectrophotometric and stopped flow kinetic studies on the formation of reduced flavoprotein intermediates. J Biol Chem **254**: 7097–7104.