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 32P-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.
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 antitumor 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 cova- lent 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-ylidum and ellipticine-13-ylidum, 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,
Chloroform, glutathione, 4-(2-hydroxyethyl)-1-piperazinoethanesulfonic 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 32P-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 32P-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 (pH7.4), 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 32P-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-hydroxyellipticine (Stiborova et al. 2007a) (C), ellipticine N2-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 μM 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 20 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 32P-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 polyethyleneimine-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 endoplasmatic reticulum (i.e., microsomal epoxide hydrolase, cytochrome b5 and its reductase, NADH:cytochrome b5 reductase). But their levels were, in comparison to overexpressed 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 32P-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 32P-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).](http://node.nel.edu)
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 the natural nanoparticle system of Supersomal microsomes. Nevertheless, the artificially prepared liposome nanoparticles with CYP3A4 catalyzed activation of 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 preparedosome 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


