ORIGINAL ARTICLE

Isolation and partial characterization of the adduct formed by 13-hydroxyellipticine with deoxyguanosine in DNA

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Abstract

OBJECTIVES: Ellipticine is a potent antineoplastic agent exhibiting multiple mechanisms of its action. Recently, we have found that 13-hydroxyellipticine, formed from ellipticine as the predominant metabolite in human livers, is bound to deoxyguanosine in DNA, generating the major DNA adduct *in vivo* and *in vitro*. The development of the methods suitable for the preparation of this adduct in the amounts sufficient for identification of its structure and those for its isolation and partial characterization is the aim of this study.

METHODS: High performance liquid chromatography (HPLC) was employed for separation of 13-hydroxyellipticine-mediated deoxyguanosine adduct. The ^{32}P-postlabeling technique was utilized to detect this adduct in DNA.

RESULTS: The formation of the 13-hydroxyellipticine-derived deoxyguanosine adduct in DNA *in vitro* was increased under the alkaline pH of the incubations and by the formation of the sulfate and acetate conjugates of 13-hydroxyellipticine generated by reactions with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) or acetyl-coenzyme A (acetyl-CoA) catalyzed by human sulfotransferases (SULTs) 1A1 and 1A2 and *N,O*-acetyltransferases (NATs) 1 and 2. The HPLC method suitable for separation the 13-hydroxyellipticine-derived deoxyguanosine adduct from other reactants, deoxyguanosine and 13-hydroxyellipticine, was developed. The structure of this adduct is proposed to correspond to the product formed from ellipticine-13-ylium with the exocyclic 2-NH_2 group of guanine in DNA.

CONCLUSIONS: The data are the first report on HPLC isolation of the deoxyguanosine adduct formed by 13-hydroxyellipticine in DNA and its partial characterization.

Abbreviations

Acetyl-CoA -acetyl-coenzyme A
CYP - cytochrome P450
HPLC - high performance liquid chromatography
NAT - *N,O*-acetyltransferase
PAPS - 3′-phosphoadenosine-5′-phosphosulfate
RAL - relative adduct labeling
r.t. - retention time
SULT - sulfotransferase

INTRODUCTION

Ellipticine (Figure 1), an alkaloid isolated from Apocynanacea plants, exhibits significant antitumor activities (for a summary, see [10]). Ellipticine has been reported to arrest cell cycle progression, to induce apoptotic cell death by the generation of cytotoxic free radicals, an increase of wild-type p53, the rescue of mutant p53 activity and the initiation of mitochondrial pathway (for a summary, see [15]). Chemotherapy-induced cell cycle arrest was shown to result from various DNA damages caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA [1,15] and (ii) inhibition of DNA topoisomerase II activity [1,15]. 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 [7,9–11,13,14,16,17], suggesting a third possible mechanism of action.

Human and rat CYP1A, 1B1 and 3A are the predominant enzymes catalyzing oxidation of ellipticine *in vitro* either to metabolites that are excreted (7-hydroxy- and 9-hydroxyellipticine) or that form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) [6,10,11,16,17]. Of the mammalian peroxidases, cyclooxygenase-1 and -2, lactoperoxidase and myeloperoxidase efficiently generated ellipticine-derived DNA adducts (Figure 1) [7,13]. The same DNA adducts were also detected in cells in culture expressing enzymes activating ellipticine (CYP1A1, cyclooxygenase-1 and myeloperoxidase), such as human breast adenocarcinoma MCF-7 cells [2], leukemia HL-60 and CCRF-CEM cells [8] and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 [5]. 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.

13-Hydroxyellipticine formed as the predominant metabolite by CYP3A4 in human livers was identified to bind to deoxyguanosine in DNA, generating the major DNA adduct *in vitro* and *in vivo* (Figure 1) [6,9,11,13,16,17]. We have suggested earlier that the reactive carbenium ion formed spontaneously from 13-hydroxyellipticine, ellipticine-13-ylium, might react with one of the nucleophilic centers in the deoxyguanosine residue in DNA (e.g. the exocyclic amino group of guanine, Figure 1) to form the adduct [7,9,13,16]. The low amount of this DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine, however, prevented its further structural characterization. Therefore, to prepare this adduct in amounts sufficient for its further characterization, we investigated how to increase its formation *in vitro*. To increase the levels of the 13-hydroxyellipticine-derived DNA adduct, we investigated the modulation of the reaction of 13-hydroxyellipticine with DNA (or deoxyguanosine) by pH and/or by its conjugation with PAPS or acetyl-CoA catalyzed by the phase II biotransformation enzymes, human SULT1A1/2 and NAT1 and NAT2. Moreover, the HPLC isolation procedure to obtain the adduct was developed.

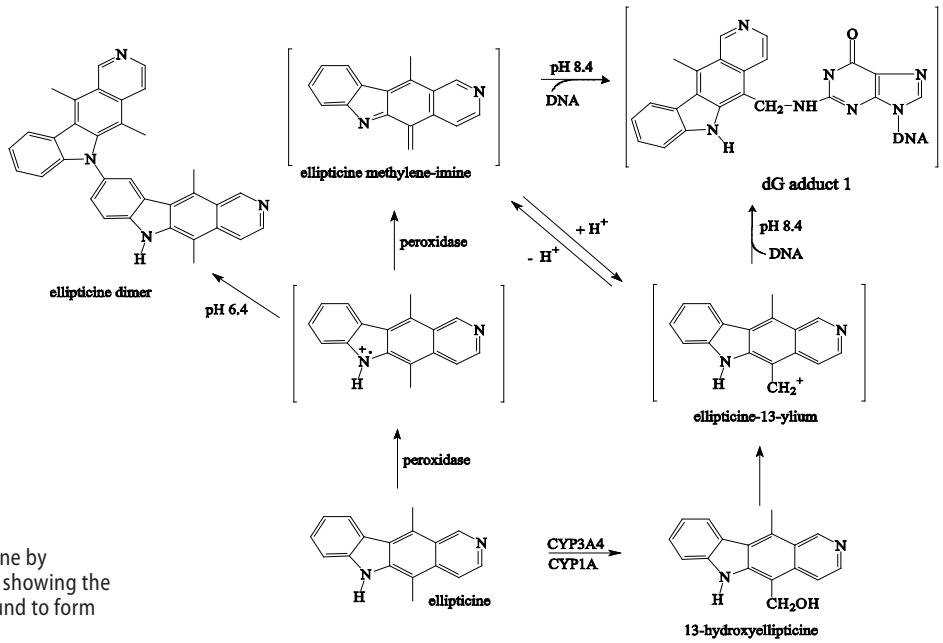

Figure 1. Metabolism of ellipticine by human CYPs and peroxidases showing the characterized metabolites found to form the major DNA adduct

MATERIAL AND METHODS

Ellipticine, deoxyguanosine, acetyl-CoA, PAPS and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA). 13-Hydroxyellipticine was synthesized as described [4]. Cytosolic extracts, isolated from insect cells transfected with baculovirus constructs containing cDNA of human SULT1A1*2, -1A2*1 were obtained from Oxford Biomedical Research Inc. (Oxford, MA, USA), and those containing cDNA of human NAT1*4 or NAT2*4 from Gentest Corp. (Woburn, MA, USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described [10,12]. All these and other chemicals were reagent grade or better. The incubation mixtures in a final volume of 500 µl consisted of 0.1 mM phosphate buffer (pH 6.0, 7.4 or 8.4), 1 mg calf thymus DNA or 1 mM deoxyguanosine and 50 µM 13-hydroxyellipticine either in the presence or absence of 39 nmol human SULT1A1/2 or NAT1 or 2 and SULTs and NATs cofactors (2 mM acetyl-CoA or 100 µM PAPS). Mixtures were incubated at 37 °C for 1–24 h. Aliquots of the mixtures (50 µl) were applied onto a HPLC column, where components of the incubation mixtures were separated. The HPLC was performed with a reversed phase column (Ultrasphere, ODS, 250 × 4.6 mm, 5 µM; Beckman-Coulter, USA) using a linear methanol – acetic acid (32 mM in distilled water) gradient of 0% methanol rising to 100% in 70 min and isocratic elution of 100% methanol in 5 min (flow rate of 1 ml/min, detection at 250 and 318 nm). Deoxyguanosine, 13-hydroxyellipticine and the 13-hydroxyellipticine-derived deoxyguanosine adduct were eluted with retention times (r.t.) of 10.0, 42.2 and 23.8 min, respectively, and identified by mass spectroscopy. ^{32}P-postlabeling assays of the 13-hydroxyellipticine-mediated adduct were performed using nuclease P1 enrichment [10].

Table 1. The effect of pH on levels of the adduct formed by reaction of 13-hydroxyellipticine with DNA[a], detected by ^{32}P-postlabeling[b]

pH	RAL[c] (mean ± SD/10^7 nucleotides)
6.0	1.3 ± 0.3
7.4	23.3 ± 2.0
8.4	49.7 ± 3.2

[a]See adduct spot 1 in Figure 2. [b]Experimental conditions were as described in Material and methods excerpt that 50 µM 13-hydroxyellipticine and 1 mg calf thymus DNA (37 °C, 90 min) were used. [c]RAL, relative adduct labeling, and standard deviations were obtained from triplicate determinations.

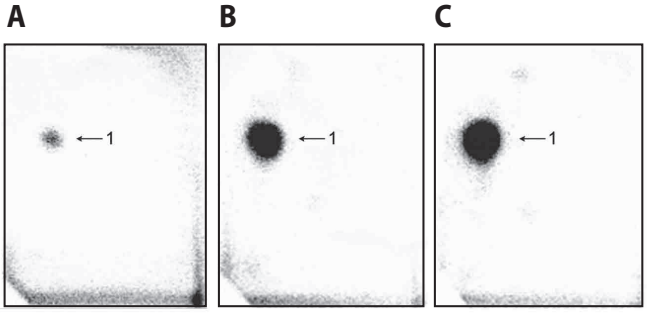

Figure 2. Autoradiographic profiles of 13-hydroxyellipticine-derived DNA adduct levels in relation to pH, analyzed with ^{32}P-postlabeling assay. (**A**) pH 6.0; (**B**) pH 7.4; (**C**) pH 8.4.

RESULTS

The effect of pH on formation of deoxyguanosine adduct from 13-hydroxyellipticine in DNA

13-Hydroxyellipticine incubated with DNA *in vitro* generates the major deoxyguanosine adduct, which was detected and quantified by the nuclease P1 version of the ^{32}P-postlabeling technique (see adduct spot 1 formed in DNA in Figure 2). The yield of formation of this ellipticine-DNA adduct is pH-dependent. Only low levels of this DNA adduct was detectable at pH 6.0, while increasing pH resulted in a pronounced increase in formation of this adduct (Figure 2, Table 1). The 13-hydroxyellipticine-derived adduct is also formed by incubation of 13-hydroxyellipticine with deoxyguanosine (Figure 3). In order to isolate the 13-hydroxyellipticine-derived deoxyguanosine adduct, a novel HPLC procedure was developed. The reversed-phase HPLC was found to be appropriate to isolate this adduct from residual deoxyguanosine and 13-hydroxyellipticine, the adduct eluted with r.t. of 23.8 min (Figure 3). An increase in pH of the incubation mixture leads again to an increase in formation of this adduct. Whereas adduct was formed at pH 8.4, no detectable levels of this adduct were found at pH 7.4 (Figure 3).

The effect of conjugation of 13-hydroxyellipticine with PAPS and acetyl-Co A catalyzed by SULT1A1/2 and NAT1/2 on formation of 13-hydroxyellipticine-derived DNAadduct

The levels of 13-hydroxyellipticine-derived DNA adduct were significantly increased by incubation of 13-hydroxyellipticine and DNA with the human SULT1A1 and 1A2 conjugation enzymes and their cofactor, PAPS, by 1.7- and 26-fold, respectively (Figure 4). Likewise, NAT1 and NAT2 in the presence of their cofactor, acetyl-CoA, stimulated the formation of the 13-hydroxyellipticine-derived DNA adduct, by 33- and 288-fold (Figure 4). An increase in its formation was detected both by the ^{32}P-postlabeling assay (Figure 4) and by HPLC (see the adduct peak eluted at 23.8 min in Figure 5).

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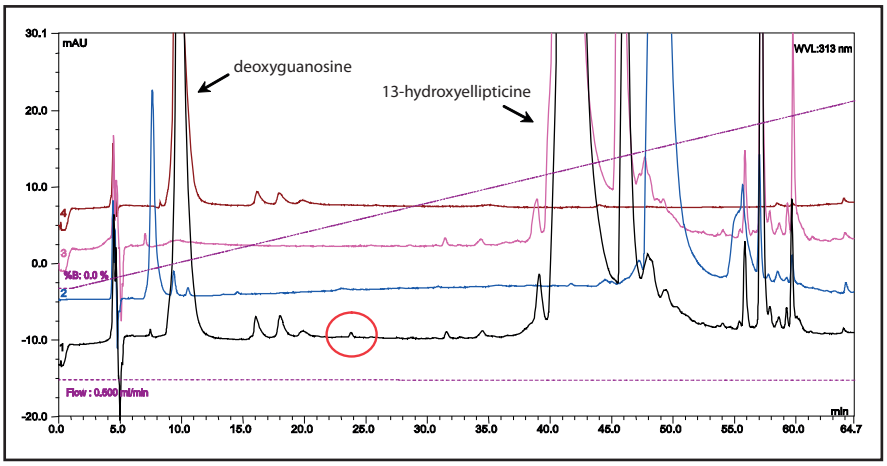

Figure 3. HPLC of 13-hydroxyellipticine-derivated DNA adduct formed by incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 and 8.4
1 – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 8.4; **2** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4; **3** – 13-hydroxyellipticine; **4** – deoxyguanosine.

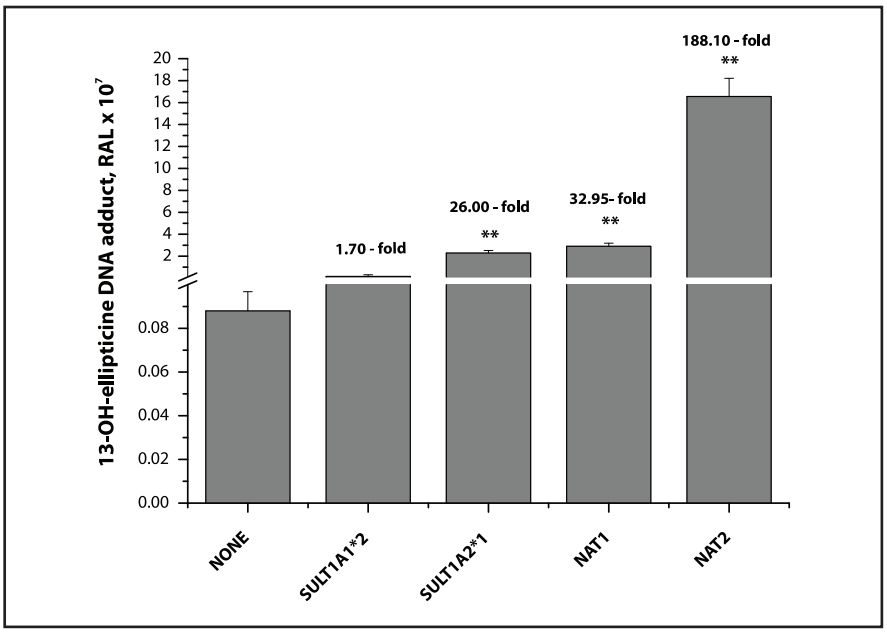

Figure 4. The effect of SULT1A1/2 and NAT1 and 2 on formation of deoxyguanosine adduct in DNA by its incubation with 13-hydroxyellipticine

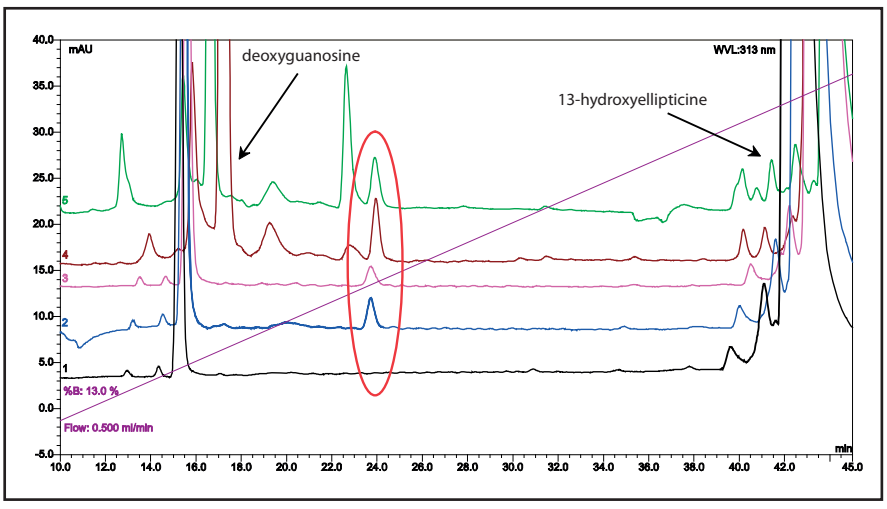

Figure 5. HPLC of 13-hydroxyellipticine-derivated DNA adduct formed by incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 in the presence of SULT 1A1/2 and NAT1 and NAT2 in the presence of their cofactors, PAPS and acetyl-CoA, respectively.
1 – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4; **2** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with PAPS and SULT1A1;
3 – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with PAPS and SULT1A2; **4** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with acetyl-CoA and NAT1;
5 – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with acetyl-CoA and NAT2.

DISCUSSION

The present paper shows the results which might increase our knowledge on the mechanism of DNA adduct formation by the anticancer drug ellipticine. Here, we demonstrate that the formation of the major DNA adduct by ellipticine *in vitro* and *in vivo*, 13-hydroxyellipticine-derived DNA adduct, is increased by an increase in pH of the incubation mixture. This adduct was proposed to be formed from the reactive species, carbenium ion (ellipticine-13-ylium), formed in the ellipticine oxidation with CYPs and peroxidases (through 13-hydroxyellipticine and/or ellipticine methylene-imine) [13] (Figure 1). Such a species was proposed to react with one of the nucleophilic centers in the deoxyguanosine residue (i.e. the 2-NH_2 group of guanine) in DNA [9,12]. The finding that the levels of this deoxyguanosine adduct significantly decreased under acidic conditions strongly supported the above suggestion. A decrease in pH leads to protonation of the NH_2 group of guanine in the DNA chain, causing a decrease in its nucleophilicity, essential for binding of ellipticine-13-ylium. The basic pH might also facilitate the second electron transfer to form the ellipticine methylene-imine (Figure 1).

The formation of the 13-hydroxyellipticine-derived DNA adduct was also significantly increased by conjugation of 13-hydroxyellipticine with PAPS or acetyl-CoA to the sulfate and acetate esters catalyzed by SULTs and NATs. This finding might have physiological significance. Some of these conjugation enzymes were found to be expressed in the target tumors for ellipticine action (e.g. human breast cancer) [18]. Therefore, by stimulation of the formation of the 13-hydroxyellipticine-derived DNA, by 13-hydroxyellipticine conjugation to sulfate and acetate esters, the pharmacological efficiency of ellipticine should be increased.

We can conclude that the results found in this work support the proposed mechanism of the reaction responsible for formation of the major deoxyguanosine adduct formed in DNA by ellipticine (Figure 1). Ellipticine is bound to deoxyguanosine by its 13-methyl group, which is activated after hydroxylation due to CYP-mediated oxidation to alcohol (13-hydroxyellipticine). Namely, this hydroxylated methyl group either alone or as the sulfate or acetate ester acts as the precursor of the vinylogous imine intermediate or the carbenium ion. Michael-type addition of the intermediates to external amino group of deoxyguanosine then leads to formation of the adduct found in DNA [3]. The study targeted to confirm this suggestion is under way in our laboratory.

ACKNOWLEDGMENT

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REFERENCES

1 Auclair C. Multimodal action of antitumor agents on DNA: The ellipticine series. Arch Biochem Biophys 1985; **259**: 1–14.
2 Bořek-Dohalská L, Frei E., Stiborová M. DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. Collect Czech Chem Commun 2004; **69**: 603–615.
3 Donaghy M.J., Stanforch S.P. Nucleophilic addition of fused benzimidazole *N*-oxide. Tetrahedron 1999; **55**: 1441–1448.
4 Dračínský M., Sejbal J., Rygerová B., Stiborová M. An efficient modification of ellipticine synthesis and preparation of 13-hydroxyellipticine. Tetrahetron Lett 2007; **48**: 6893–6895.
5 Frei E, Bieler C, Arlt VM, Wiessler M, Stiborová M. Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. Biochem Pharmacol 2002; **64**: 289–295.
6 Kotrbová V., Aimová D., Březinová A., Janouchová K.., Poljaková J., Hodek P., Frei E., Stiborová M. Cytochromes P450 reconstituted with NADPH: P450 reductase mimic the activating and detoxicating metabolism of the anticancer drug ellipticine in microsomes. Neuro Endocrinol Lett 2006; **27** (Suppl. 2): 18–20.
7 Poljaková J, Dračínský M, Frei E, Hudeček J, Stiborová M. The effect of pH on peroxidase-mediated oxidation of and DNA adduct formation by ellipticine. Collect Czech Chem Commun 2006; **71:** 1169–1185.
8 Poljaková J., Frei E., Gomez J.E., Aimová D., Eckschlager T., Hraběta J., Stiborová M. DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. Cancer Lett 2007; **252**: 270–279.
9 Stiborová M., Arlt V.M., Henderson C.J., Wolf C.R., Kotrbová V., Moserová M., Hudeček J., Phillips D.H., Frei E. 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 2008; **226:** 318–327.
10 Stiborová M, Bieler CA, Wiessler M, Frei E. The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. Biochem Pharmacol 2001; **62**: 1675–1684.
11 Stiborová M, Breuer A, Aimová D, Stiborová-Rupertová M, Wiessler M, Frei E. DNA adduct formation by the anticancer drug ellipticine in rats determined by ^{32}P-postlabeling. Int J Cancer 2003; **107**: 885–890.
12 Stiborová M, Martínek V, Schmeiser HH, Frei E. Modulation of CYP1A1-mediated oxidation of carcinogenic azo dye Sudan I and its binding to DNA by cytochrome b_5. Neuro Endocrinol Lett 2006; **27**(Suppl. 2): 35–39.
13 Stiborová M, Poljaková J, Ryšlavá H, Dračínský M, Eckschlager T, Frei E. Mammalian peroxidases activate 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 2007; **120**: 243–251.
14 Stiborová M., Rupertová M., Aimová D., Ryšlavá H., Frei E. Formation and persistence of DNA adducts of anticancer drug ellipticine in rats. Toxicology 2007; **236**: 50–60.
15 Stiborová M, Rupertová M, Schmeiser HH, Frei E. Molecular mechanism of antineoplastic action of an anticancer drug ellipticine. Biomed. Pap 2006; **150**: 13–23.
16 Stiborová M, Sejbal J, Bořek-Dohalská L, Aimová D, Poljaková J, Forsterová K, Rupertová M, Wiesner J, Hudeček J, Wiessler M, Frei E. 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 2004; **64**: 8374–8380.
17 Stiborová M, Stiborová-Rupertová M, Bořek-Dohalská L, Wiessler M, Frei E. Rat microsomes activating the anticancer drug ellipticine to species covalently binding to deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. Chem Res Toxicol 2003; **16**: 38–47.
18 Williams J.A., Phillips D.H. Mammary expression of xenobiotic metabolizing enzymes and their potential role in breast cancer. Cancer Res 2000; **60**: 4667–77.

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