

INTRODUCTION

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) and several of its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N²-methyl-ellipticinium, 9-chloro-N²-methyl-ellipticinium and 9-methoxy-N²-methyl-ellipticinium) exhibit promising results in the treatment of osteolytic breast cancer metastases, tumors of brain and myeloblastic leukemia (Stiborova *et al.*, 2001). The main reason for the interest in ellipticines for clinical purposes is their high efficiency against several types of cancer, their rather limited toxic side effects and their complete lack of hematological toxicity (Auclair, 1987). Nevertheless, ellipticines are potent mutagens (Stiborova *et al.*, 2001).

The antineoplastic property of ellipticine was considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II (Monnot *et al.*, 1991). Recently, it was found that ellipticine covalently binds to DNA after being enzymatically activated (Stiborova *et al.*, 2001). The major enzymes catalyzing the ellipticine oxidation and its activation to more efficient metabolite(s) forming DNA adducts are cytochromes P450 (CYPs). Deoxyguanosine was identified as the target base, to which the ellipticine reactive metabolites generated by CYPs are bound, forming the two major ellipticine-derived DNA adducts *in vitro* and *in vivo* (Stiborova *et al.*, 2003a; Stiborova *et al.*, 2003b). These deoxyguanosine adducts with ellipticine were detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (Frei *et al.*, 2002), in human breast adenocarcinoma MCF-7 cells (Bořek -Dohalská *et al.*, 2004) and in rats exposed to this anticancer drug (Stiborova *et al.*, 2003a). Besides these two major adducts, up to four minor adduct spots were detectable in DNA reacted with ellipticine activated with CYPs (Stiborova *et al.*, 2001; Stiborova *et al.*, 2003a), or in V79 (Frei *et al.*, 2002) and MCF-7 cells (Bořek -Dohalská *et al.*, 2004) exposed to ellipticine or *in vivo* (Stiborova *et al.*, 2003a). Based on these data, ellipticine

might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues.

While CYP enzymes are expressed in breast and kidney cancer (Murray *et al.*, 1993), their levels are much lower in some other tumor cells (i.e. leukemia cells) that are sensitive to ellipticine. The question thus arises, which other enzymes are involved in ellipticine activation in these malignant tissues. Peroxidases expressed in some cancer cells might be good candidates for such ellipticine activation (Soslow *et al.*, 2000; Matsuo *et al.*, 2001).

Our preliminary studies demonstrated that ellipticine is oxidized by peroxidases (Poljaková, 2002). This process has not yet been characterized in detail, but this knowledge is important to evaluate the pharmacological efficacy and/or the genotoxic side effects of ellipticine in organisms, including humans.

AIM OF THE STUDY

The present study was undertaken to extend our knowledge on:

- the ellipticine oxidation by peroxidases and/or cytochromes P450, and
- the mechanism of formation of ellipticine-derived DNA adducts generated during such enzymatically catalyzed oxidations *in vitro* or in leukemia and neuroblastoma cancer cells in culture.

The target of present work was mainly:

- (i) To determine the capability of different peroxidases [human myeloperoxidase (MPO), human and ovine prostaglandin H synthase (cyclooxygenase, COX), bovine lactoperoxidase (LPO), and plant HRP] to oxidize ellipticine and characterize peroxidase-mediated ellipticine metabolites. To compare the pattern of ellipticine metabolites generated by peroxidases and cytochromes P450.
- (ii) To determine whether during peroxidase-mediated ellipticine oxidation DNA adducts are generated, and to identify which ellipticine reactive species form adducts in DNA.
- (iii) To determine the cytotoxicity of ellipticine to human leukemia HL-60 and CCRF-CEM cells and neuroblastoma cell lines IMR-32, UKF-NB-3 and UKF-NB-4 and its ability to generate DNA adducts in these cells. Beside the parental neuroblastoma cell lines, those resistant to Vincristine, Doxorubicin and Cis-platin were also tested for their sensitivity to ellipticine and capabilities of ellipticine-DNA adduct formation.
- (iv) To investigate the effect of inhibitors of histone deacetylases, valproate and trichostatin A, on toxicity of ellipticine to human neuroblastoma UKF-NB-3 and UKF-NB-4 cells.

RESULTS AND DISCUSSION

The results shown in the dissertation demonstrate that peroxidases such as bovine LPO, human MPO, ovine COX-1, human COX-2 and plant HRP oxidize the anticancer drug ellipticine to species binding to DNA. Using two independent methods, ^{32}P -postlabeling and ^3H -labeled ellipticine, we showed that ellipticine binds covalently to DNA *in vitro* after its oxidation by the peroxidases. During the ellipticine oxidation by peroxidases, two ellipticine metabolites, ellipticine dimer and ellipticine N^2 -oxide, were detectable under the conditions used in the experiments. The major one was characterized by NMR spectroscopy to be the ellipticine dimer, where two ellipticine residues are connected *via* the nitrogen atom N^6 in the pyrrole ring of one of the ellipticine molecules and the carbon atom C9 of the second ellipticine. It should be noted that the dimer product is much less likely to be formed physiologically than in this experimental system, because no other molecules (bionucleophiles) are present there to intercept ellipticine reactive intermediates. Indeed, during the ellipticine oxidation by peroxidases in the presence of DNA up to four DNA adducts are formed, and an increase in the formation of the major DNA adduct I correlates with a decrease in generation of the ellipticine dimer. Hence, ellipticine-DNA adduct formation seems to be the preferential reaction under the physiological conditions.

CYP enzymes oxidize ellipticine to metabolites containing one atom of oxygen in their molecules; 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2 -oxide are generated by this enzymatic system. Two of the ellipticine metabolites generated by human CYP enzymes, 13-hydroxy- and 12-hydroxyellipticine (the latter one formed also spontaneously from another ellipticine metabolite, ellipticine N^2 -oxide, by Polonowski rearrangement), are responsible for formation of the two major deoxyguanosine DNA adducts (Fig. 1). Even though oxidation of ellipticine catalyzed by CYPs

is not identical with that catalyzed by peroxidases (only ellipticine N^2 -oxide is generated by CYPs and peroxidases), both enzymes generate the same two major DNA adducts, found to be formed also *in vivo*, in rats treated with ellipticine.

Even though two-electron-oxidation metabolite such as methylene-imine (Fig. 1) was not found to be formed from ellipticine by peroxidases under the conditions used in our experiments, we suggest that such an ellipticine metabolite is responsible for the formation of the ellipticine-derived DNA adduct I. As mentioned above and shown in Figure 1, this adduct is generated from 13-hydroxyellipticine. Because of the reactivity of this compound, we can suggest that it might, depending on the environment, decompose spontaneously to the reactive carbenium ion, ellipticine-13-ylum, which reacts with one of the nucleophilic centers in the deoxyguanosine residue in DNA (i.e. the exocyclic NH_2 group of guanine). The identity of the adduct I generated from 13-hydroxyellipticine with that formed by peroxidases allow us to estimate the structure of a two-electron oxidation metabolite of ellipticine responsible to its formation as the methylene-imine derivative of ellipticine. It namely forms the same carbenium ion, ellipticine-13-ylum, as 13-hydroxyellipticine (Fig. 1).

The minor ellipticine oxidation product, the N^2 -oxide, was identified as a metabolite that is also generated by human CYP enzymes. In this activation system an N^2 -oxidation of ellipticine was found to be the prerequisite for the formation of the second deoxyguanosine adduct 2 in DNA. The identical DNA adduct was generated from ellipticine after its activation by peroxidases. Moreover, we proved that this adduct is in fact generated from 12-hydroxyellipticine, which is formed from ellipticine N^2 -oxide spontaneously (Fig. 1), by the Polonowski rearrangement. As in the case of 13-hydroxyellipticine, we postulate another carbenium ion (ellipticine-12-ylum), reacting with the nucleophilic centres of deoxyguanosine in DNA, and generating the DNA adduct 2 (Fig. 1).

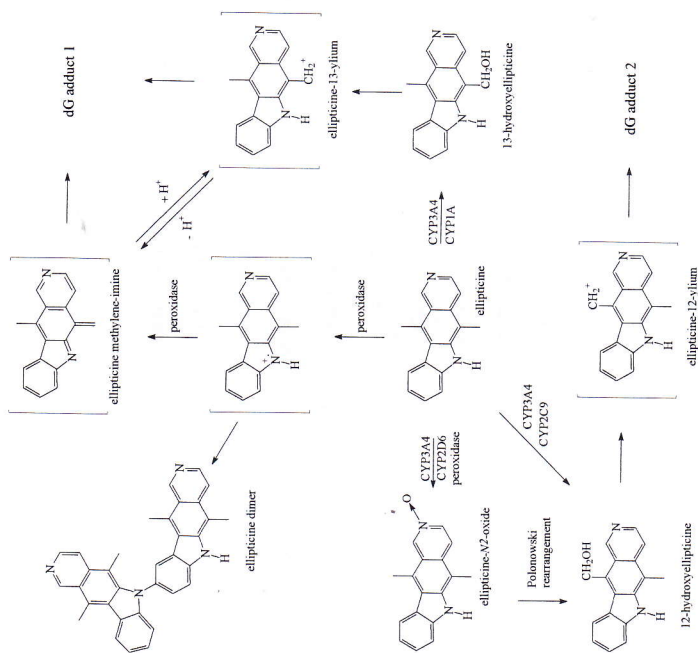


Figure 1

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

We found that the formation of DNA adducts by ellipticine activated with peroxidases is not dictated only by the optimal conditions (pH) for ellipticine oxidation by these enzymes. While acidic pH facilitates ellipticine oxidation by peroxidases, an increase in pH resulted in the pronounced increase in DNA adduct formation. The most significant sensitivity to the changes in pH was found for formation of the major ellipticine-derived DNA adduct (adduct 1). The finding that formation of the deoxyguanosine adduct 1 (supposed to be

generated by ellipticine reactive species, ellipticine-13-ylum) is significantly decreased under the acidic conditions strongly supported the suggestion on its binding to the exocyclic $-NH_2$ group of guanine in DNA. Namely, a decrease in pH leads to protonation of the $-NH_2$ group of guanine in the DNA chain, finally causing a decrease in its nucleophilicity, essential for ellipticine-13-ylum binding.

The results found in the present work demonstrate that ellipticine is toxic to two model leukemia cell lines, HL-60 and CCRF-CEM cells in culture. In addition, after exposition of the cells to this drug, ellipticine-derived DNA adducts are generated. Cytotoxicity of ellipticine towards the leukemia cells investigated in our laboratory correlates with levels of ellipticine-derived DNA adducts generated in these cells. Because HL-60 cells, in contrast to CCRF-CEM cells, expressed high levels of MPO, this enzyme (found to activate ellipticine to species forming DNA adducts) might therefore be responsible for higher DNA adduct formation in HL-60 cells. These results and the fact that ellipticine is activated by several peroxidases *in vitro* are interesting findings, which might explain the ellipticine cytotoxic activity against human leukemia.

Additional results presented in this dissertation are the first report showing the cytotoxicity of ellipticine towards human neuroblastoma cells (human parental IMR-32, UKF-NB-3 and UKF-NB-4 cell lines) and their daughter derived cells that were resistant to Vincristine, Doxorubicine and Cisplatin. In addition, ellipticine generates covalent DNA adducts in these human cancer cell lines. The results indicate the formation of covalent DNA adducts by ellipticine as one of the multiple modes of antitumor action of ellipticine for these cancer cells.

The pre-treatment of UKF-NB-3 and UKF-NB-4 neuroblastoma cells with inhibitors of histone deacetylase, valproate and trichostatin A, resulted in an increase in the toxicity of ellipticine to these cells. The increase in toxicity of ellipticine to UKF-NB-3 and UKF-NB-4 cell lines caused by both compounds

was dose-dependent and correlated with an increase in ellipticine-DNA adduct formation, predominantly in UKF-NB-3 cells (Fig. 2). Moreover, UKF-NB-3 neuroblastoma cells were more sensitive to valproate and trichostatin A than the UKF-NB-4 line.

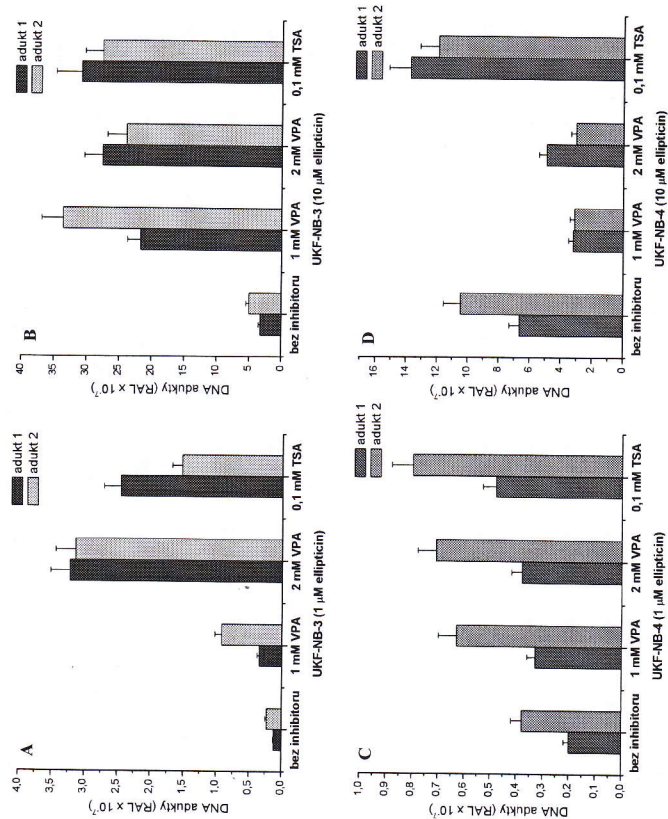


Figure 2
The effect of valproate (VPA) and trichostatin A (TSA) on DNA adduct formation by ellipticine in neuroblastoma cells. Cells were treated with VPA and TSA 24 h before their exposition to ellipticine

Even though the mechanism of valproate and trichostatin A effects on toxicity of ellipticine to neuroblastoma cells remains to be elucidated, the finding that both compounds stimulate the ellipticine anticancer efficiency is promising result for their potential use in human anticancer therapy.

Results present in this dissertation were published in the original papers and as short communications or abstracts (see list of publications).

The work was supported by the Grant Agency of the Czech Republic (grant 203/06/0329) and Ministry of Education of the Czech Republic (grants MSM0021620808 and MSM0021620813).