Int. J. Electrochem. Sci., 9 **(2014)** 5675 - 5689

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Expression Levels of Enzymes Metabolizing an Anticancer Drug Ellipticine Determined by Electromigration Assays Influence its Cytotoxicity to Cancer Cells - A Comparative Study

Marie Stiborova[1,*], *Jitka Poljakova*[1], *Iveta Mrizova*[1], *Lucie Borek-Dohalska*[1], *Tomas Eckschlager*[2], *Vojtech Adam*[3,4], *Rene Kizek*[3,4], *Eva Frei*[5]

[1] Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic
[2] Department of Pediatric Hematology and Oncology, 2nd Medical School, Charles University and University Hospital Motol, V Uvalu 84, CZ-150 06 Prague 5, Czech Republic
[3] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[4] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic
[5] Division of Preventive Oncology, National Center for Tumor Diseases, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, D-69 120 Heidelberg, Germany
[*]E-mail: stiborov@natur.cuni.cz

Received: 27 May 2014 / *Accepted:* 23 June 2014 / *Published:* 16 July 2014

The antineoplastic alkaloid ellipticine is a prodrug, of which the pharmacological efficiency is dependent on its cytochrome P450 (CYP)- and/or peroxidase-mediated activation to 12-hydroxy- and 13-hydroxyellipticine, which are both the metabolites forming DNA adducts in target tissues. Using the method of Western blotting, the expression of CYP1A1, 1B1, 3A4, lactoperoxidase, thyroid peroxidase, cyclooxygenase-1 and cytochrome b_5, the enzymes that catalyze and/or influence ellipticine metabolism, was investigated in several cancer cells sensitive to ellipticine (HL-60 promyelocytic leukemia and T-cell leukemia CCRF-CEM cells, glioblastoma U87MG cells, thyroid cancer BHT-101, B-CPAP and 8505-C cells, neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines and breast adenocarcinoma MCF-7 cells). The findings summarized from several former studies reviewed in this study, together with new results indicate that, depending on individual cells, cytotoxicity of ellipticine, which is mediated by formation of covalent DNA adducts to these cancer cells, is influenced by expression levels of these CYP and peroxidase enzymes in the tested cancer cells. Furthermore, a potency of ellipticine to induce the enzymes dictating activation of ellipticine to form DNA adducts in studied cancer cells determines an increase in cytotoxicity of ellipticine to these tumor cells.

Keywords: Ellipticine; Cancer Cells; Cytotoxicity; Cytochromes P450; Peroxidases; Protein Expression; Western Blotting; Metabolism; DNA Adducts

1. INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1) and its derivatives are efficient anticancer compounds that function through multiple mechanisms participating in cell cycle arrest and initiation of apoptosis (for a summary see [1-6]). Ellipticine was found (i) to arrest cell cycle progression due to modulation of levels of cyclinB1 and Cdc2, and phosphorylation of Cdc2 in human mammary adenocarcinoma MCF-7 cells, (ii) to initiate apoptosis due to formation of toxic free radicals, stimulation of the Fas/Fas ligand system and modulation of proteins of Bcl-2 family in several tumor cell lines, and (iii) to induce the mitochondria-dependent apoptotic processes (for a summary see [3,4]). The predominant mechanisms of ellipticine's biological effects were suggested to be (i) intercalation into DNA [5-7] and (ii) inhibition of topoisomerase II [3-6]. Further, we showed that this antitumor agent forms covalent DNA adducts after its enzymatic activation with cytochromes P450 (CYP) and peroxidases [1-4,8-13], 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 [3,7,9-11]. The CYP1A isoforms also efficiently form the other ellipticine metabolites, 7-hydroxy- and 9-hydroxyellipticine, which are the detoxification products (Fig. 1). Recently, we found that cytochrome b_5 alters the ratio of ellipticine metabolites formed by CYP1A1, 1A2 and 3A4. While the amounts of the detoxification metabolites (7-hydroxy- and 9-hydroxyellipticine) were either decreased or not changed with added cytochrome b_5, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide increased considerably. The change in amounts of metabolites resulted in an increased formation of covalent ellipticine-DNA adducts, one of the DNA-damaging mechanisms of ellipticine antitumor action [11,12]. In addition, we observed that levels of the DNA adduct formed by 13-hydroxyellipticine also increased if this ellipticine metabolite was conjugated with sulfate or acetate by human sulfotransferases 1A1, 1A2, 1A3 and 2A1, or *N,O*-acetyltransferases 1 and 2 [11-14] as it is shown in Fig. 1.

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 both animal models, ellipticine-DNA adduct formation was mediated mainly by CYP1A and 3A enzymes, but a role of peroxidases in several organs has been proved [15-17]. Therefore, expression levels of CYP and peroxidase enzymes metabolizing ellipticine seem to be crucial for antitumor, cytostatic and genotoxic activities of this drug in individual tissues. The ellipticine-DNA adducts were also found in several cancer cell lines and in DNA of rat mammary adenocarcinoma *in vivo* [3,19-25]. In this study, the utilizing the findings from several former studies [19-25] as well as new results found in this work, we evaluated whether cytotoxicity of ellipticine to cancer cells is dependent on formation of covalent ellipticine-DNA adducts and whether the expression of the enzymes metabolizing ellipticine *in vitro* and *in vivo* regulates their levels in cancer cells as well as the ellipticine cytotoxicity to these cells. The method of Western blotting determined the enzyme protein expression levels, whereas the ^{32}P-postlabeling method detected and quantified DNA adducts formed by ellipticine [1-3,25-29].

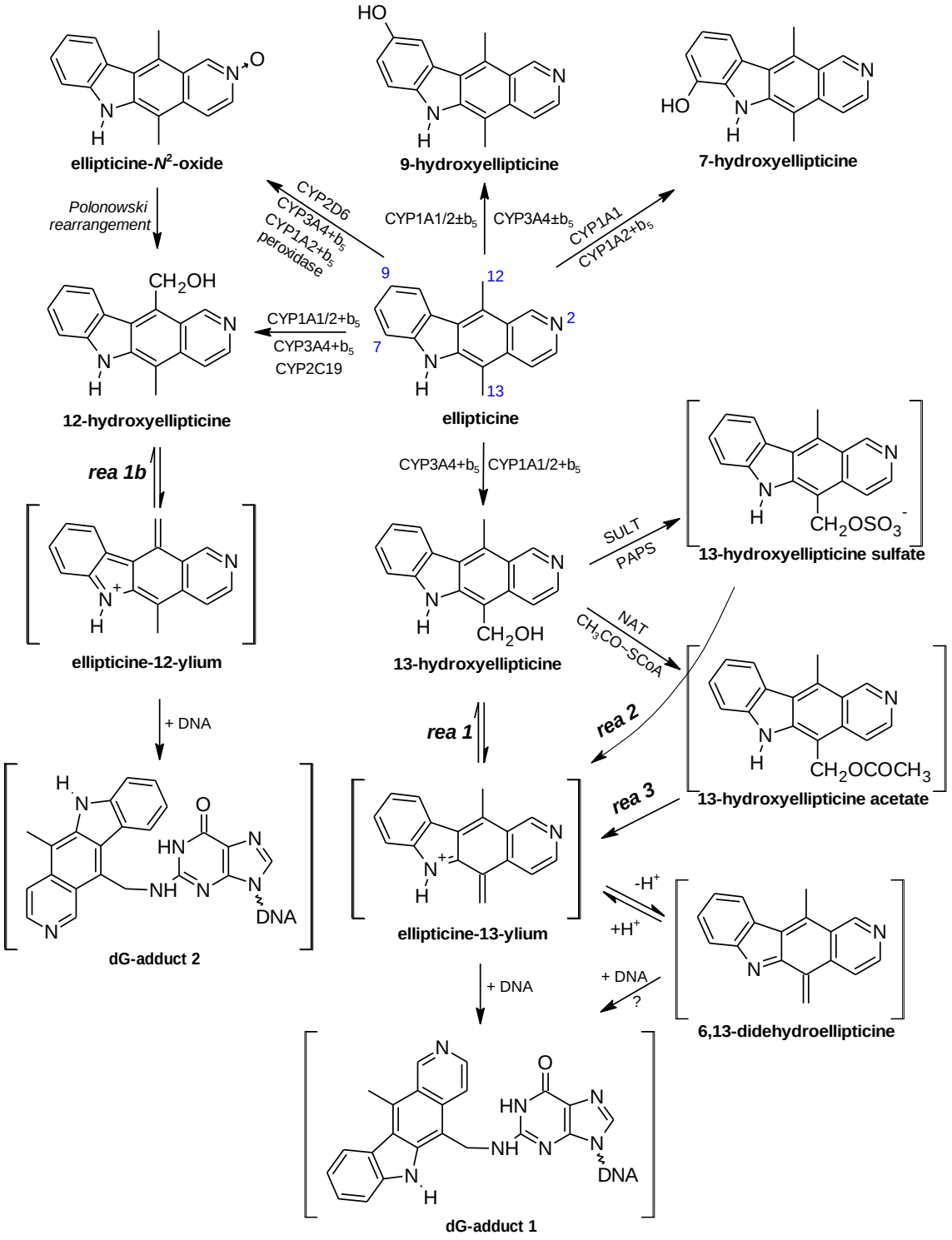

Figure 1. Scheme of ellipticine metabolism catalyzed by CYPs showing the identified metabolites and those proposed to form DNA adducts. The compounds showed in brackets were not detected under the experimental conditions and/or not yet structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in our previous studies [8,11,12]. Rea 1, 2 and 3 are reactions leading to ellipticine-13-ylium from 13-hydroxyellipticine, 13-hydroxyellipticine sulfate and 13-hydroxyellipticine acetate, respectively.

2. EXPERIMENTAL PART

2.1 Chemicals and material

Ellipticine was from Sigma Chemical Co. (St. Louis, MO, USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described [1]. All these and other chemicals used in the experiments were of analytical purity or better. 12-Hydroxy- and 13-hydroxyellipticine were isolated from multiple high performance liquid chromatography (HPLC) runs of ethyl acetate extracts of incubations containing ellipticine and human and/or rat hepatic microsomes as described [8].

2.2 Cell cultures

Several cancer cell lines were utilized in studies investigating cytotoxicity of ellipticine [19-25]. Namely, the CCRF-CEM, a T lymphoblastoid cell line, was kindly provided by J.J. McGuire, Roswell Park Cancer Institute, (Buffalo, NY, USA) and HL-60 cells (a promyelotic 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 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 (U) per ml of penicillin and 100 μg/ ml streptomycin (PAA,Vienna, Austria) and 0.3% (*w*/*v*) $NaHCO_3$ at 37°C, 5% CO_2 and 95% atmospheric humidity. CCRF-CEM cells were cultivated in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10% fetal calf serum (PAA Laboratories, Pasching, Austria) and 0.3% (*w*/*v*) $NaHCO_3$ at 37 °C, 5% CO_2 and 95% atmospheric humidity [20].

The MCF-7 cell line was from the collection of cell lines of the German Cancer Research Center Heidelberg, Germany). MCF-7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Biochrom AG, Berlin, Germany), high-glucose type (DMEM with 4.5 g D-glucose/l), supplemented with 4 mM L-glutamine, 25 mM HEPES Sigma, St. Louis, MO, U.S.A.), 5% fetal calf serum (Biochrom AG, Berlin, Germany) at 37 °C, 5% CO_2 and 95% atmospheric humidity [19].

The U87MG cell line was purchased from ATCC (Manassas, VA, USA). Cells were cultured in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.6 mg/mL glutamine, 200 international unit (IU)/ml penicillin, 200 IU/ml streptomycin, and 0.1 mg/ml gentamicin (PAA Laboratories, Pasching, Austria) in humidified 5% CO_2 at 37 °C [23].

The UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, established from bone marrow metastases of high risk neuroblastoma, were a gift of Prof. J. Cinatl, Jr. (J. W. Goethe University, Frankfurt, Germany). The cell line UKF-NB-4 was established from chemoresistant recurrence. The cell lines used were derived from high risk neuroblastoma with MYCN amplification, del1p and aneuploidy. Cells were grown at 37°C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 μg/ml streptomycine (PAA Laboratories, Pasching, Austria) [22].

The human thyroid cancer cell lines BHT-101 and B-CPAP were purchased from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweigh, Germany) and 8505-C cells from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Cells were grown at 37 °C and 5 % CO_2 in Iscove's modified Dulbecco's medium (IMDM) (KlinLab Ltd, Prague, Czech Republic), supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicillin and 100 μg/ml streptomycine (PAA Laboratories, Pasching, Austria). All cells were grown at 37 °C in an atmosphere of ambient air with 5 % CO_2 (74% N_2, 20 % O_2) [25].

2.3 MTT assay

The cytotoxicity of ellipticine was determined by MTT test. For a dose-response curve, solution of ellipticine in dimethyl sulfoxide (DMSO, 1 mM) was dissolved in culture medium to final concentrations of 0 – 10 μM. MCF-7 cells were, in another experiment, also cultivated in the presence of 0.1 μM ellipticine for 72 h and these cells were thereafter treated with 0 – 10 μMellipticine. Cells in exponential growth were seeded at 1 x 10^4 per well in a 96-well microplate. After incubation (48 hours) at 37 °C in 5% CO_2 saturated atmosphere the MTT solution (2 mg/ml PBS) was added, the microplates were incubated for 4 hours and cells lysed in 50% *N*,*N*-dimethylformamide containing 20% of sodium dodecyl sulfate (SDS), pH 4.5. The absorbance at 570 nm was measured for each well by multiwell ELISA reader Versamax (Molecular devices, CA, USA). The mean absorbance of medium controls was subtracted as a background. The viability of control cells was taken as 100% and the values of treated cells were calculated as a percentage of control. The IC_{50} values were calculated from at least 3 independent experiments using linear regression of the dose-log response curves by SOFTmaxPro.

2.4 Estimation of contents of enzymes biotransforming ellipticine in cancer cells

To determine the expression of enzymes metabolizing ellipticine [*i.e.*, CYP1A1, 1B1, 2B, 2E1 and 3A4, cytochrome b_5, lactoperoxidase (LPO), thyroid peroxidase (TPO) and cyclooxygenase (COX)-1 and -2 proteins], cell pellets were resuspended in 25 mM Tris-HCl buffer pH 7.6 containing 150 mM NaCl, 1% detergent NP-40 (Sigma, St. Louis, MO, USA), 1% sodium deoxycholate, 0.1 % SDS and with solution of COMPLETE (protease inhibitor cocktail tablet, Roche, Basel, Switzerland) at concentration described by the provider. The samples were incubated for 60 min on ice and centrifuged for 20 min at 14 000 g and 4 °C. Supernatant was used for additional analysis. Protein concentrations were assessed using the DC protein assay (Bio-Rad, Hercules, CA, USA) with serum albumin as a standard and 10-75 μM of extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 11% gel for analysis of CYP1A1, 1B1, 2B, 2E1 and 3A4, LPO, TPO and COX-1 and -2 protein expression, and a 17% gel for analysis of cytochrome b_5 protein expression [20-29]. After migration, proteins were transferred to a nitrocellulose membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific rabbit polyclonal anti-cytochrome b_5 (1:750, Abcam, MA, USA), anti-CYP1A1 (1:1000,

Millipore, MA, USA), anti-CYP1B1 (1:500, Abcam, MA, USA), anti-CYP3A4 (1:5000, AbD Serotec, Oxford, UK), anti-COX-1 (1:1000, Abcam, MA, USA) antibodies, to the anti-CYP2B4-, CYP2E1-, anti-LPO- and anti-COX-2-chicken polyclonal antibodies prepared as described [30], and to specific mouse monoclonal anti-TPO (2.5 □g/ml, Abcam, MA, USA) antibody overnight at 4 ^{o}C. Membranes were washed with distilled water and exposed to peroxidase-conjugated anti-IgG secondary antibodies (1:3000, Bio-Rad, Hercules, CA, USA), and the antigen-antibody complex was visualized by enhanced chemiluminiscence's detection system according to the manufacturer's instructions (Immun-Star HRP Substrate, Bio-Rad, Hercules, CA, USA) for thyroid cancer cells and by an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as dye for leukemia, glioblastoma, neuroblastoma and breast adenocarcinoma cells. Antibodies against glyceraldehyde-P-dehydrogenase (GAPDH) or actin (1:1000, Sigma, St. Louis, MO, USA) were used as loading controls [20-25].

2.5 Estimation of MPO content in HL-60 and CCRF-CEM cells

MPO was detected by flow cytometry using anti-human MPO-FITC antibody (Immunotech, Marseille, France, cat. no. 1874) as shown in our previous work [20]. Cultivation was performed in 12-well plates, three samples from every well were prepared and two wells measured. Cells were permeabilized with Fix and Perm kit (Caltag Laboratories, Burlingame, CA, USA, cat. no. GAS-004), according to the recommendation of a producer. The fluorescence intensity of at least 10,000 cells was measured by FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with 488 nm laser and list mode data were analyzed with the CellQuest software. Expression was evaluated as mean intensity of fluorescence. The fluorescence measurements were calibrated for each run by FITC-conjugated bead standards (DAKO cat. no. K0110). Results were expressed as mean of six determinations ± standard deviation [20].

2.6 Treatment of cancer cells with ellipticine for DNA adduct analyses

Cell lines were seeded 24 h prior to treatment with ellipticine at a density of 1 x 10^5 cells/ml in two 75 cm^2 culture flasks in a total volume of 20 ml of IMDM. Ellipticine was dissolved in 20 μM of DMSO, the final concentration was 0, 0.1, 1, 5 or 10 μM. After 24 h the cells were harvested after trypsinizing by centrifugation at 2000 x g for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at -20 ºC until DNA isolation. DNA was isolated and labeled as described in the next section.

2.7 DNA isolation and ^{32}P-postlabeling of DNA adducts

DNA from cells was isolated by the phenol-chloroform extraction as described [19-25,31,32]. ^{32}P-postlabeling analyses were performed using nuclease P1 enrichment as described previously [1].

Calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine [8,9], and liver DNA of rats treated with ellipticine [3,13,15] were used to compare DNA adduct spot patterns.

2.8 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.

3. RESULTS

3.1 Expression of CYP and peroxidase enzymes in human cancer cells

Using the method of Western blotting, the expression of several CYP enzymes and peroxidases were analyzed in cancer cell lines sensitive to ellipticine (HL-60 promyelocytic leukemia and T-cell leukemia CCRF-CEM cells, glioblastoma U87MG cells, neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines, thyroid cancer BHT-101, B-CPAP and 8505-C cells and breast adenocarcinoma MCF-7 cells).

Employing antibodies against COX-1 and LPO, expression of these enzymes was proved in HL-60 promyelocytic leukemia and T-cell leukemia CCRF-CEM cells [20]. Beside these peroxidases, expression of MPO protein was also found in HL-60 cells, but was proved by flow cytometry using an anti-human MPO-FITC antibody (FACS) [20]. In contrast to these peroxidases, Western blots with polyclonal antibodies raised against COX-2 and various CYPs (CYP1A1, 2B4, 2E1 and 3A4) showed that CYP1A1 only is expressed in HL-60 cells. In the case of a human CCRF-CEM cell line, no detectable expression of MPO was determined by FACS analysis. Western blot analyses of other peroxidases (COX-1 and -2) and of CYPs (CYP1A1, 2B4, 2E1 and 3A4) enzymes in CCRF-CEM cells revealed that COX-1 and low but detectable levels of CYP1A1 are expressed in these cells [20].

Using Western blot analysis with polyclonal antibodies raised against CYP1A1, 1B1 and 3A4, the protein expression of these enzymes was detected also in another cancer cell line sensitive to ellipticine, a U87MG glioblastoma cell line. Beside these CYP enzymes, peroxidases such as LPO and COX-1 were also found to be expressed in U87MG cells [23].

Because the expression of several CYP enzymes is known to be induced by ellipticine [22-25, 28,29], the question whether ellipticine might be capable of inducing CYP enzymes expressed in U87MG cells was investigated [23]. The Western blots with antibodies against CYP1A1, 1B1 and 3A4 showed that the expression of CYP1B1 and 3A4 was induced in U87MG cells treated with ellipticine . The expression of CYP1A1 was also increased, but to a lower extent. In contrast to induction of CYP enzymes by ellipticine, the protein expression levels of both peroxidases tested in this study, COX-1 and LPO, were not affected by ellipticine in U87MG cells [23].

In UKF-NB-3 and UKF-NB-4 cells, CYP1A1, 1B1 and 3A4 as well as peroxidases LPO and COX-1 were detectable by Western blotting [22]. In addition, low expression levels of cytochrome b_5, the protein playing an important role in CYP1A- and CYP3A4-mediated ellipticine metabolism

[11,12,32], were detected in both neuroblastoma cell lines [22]. In addition to analysis of the basal levels of these enzymes, the effect of ellipticine on expression of these proteins was also investigated. Similar to U87MG glioblastoma cells, ellipticine acts as an inducer of CYP1A1 and 3A4 in neuroblastoma cells, but expression of CYP1B1 as well as COX-1 and LPO were attenuated by treatment of these cells with ellipticine [22]. In the case of cytocrome b_5, its expression was induced by treatment of UKF-NB-3 cells with ellipticine, but not in UKF-NB-4 cells [22].

In thyroid cancer BHT-101, B-CPAP and 8505-C cells, expression patterns of CYP1A1, 1B1, 3A4, cytochrome b_5, COX-1 and peroxidase specifically expressed in thyroid cancer cells, thyroid peroxidase (TPO), were found to be quite complex [25]. The levels of CYP1A1 were high in all thyroid cancer cells and induced in B-CPAP and 8505-C cells by their treatment with ellipticine. In contrast, expression of TPO was so low that the influence of cell lineage or ellipticine exposure cannot be assessed. Expression levels of CYP1B1, COX-1 and cytochrome b_5 were different in the three thyroid cancer cell lines and attenuated by their exposure to ellipticine. Most conspicuous was the near complete absence of cytochrome b_5 in 8505-C cells and the highest induction of CYP3A4 by ellipticine in B-CPAP cells [25].

In human breast adenocarcinoma MCF-7 cells, only the expression of CYP1A1, 1B1 and 3A4 enzymes was analyzed in this work, whereas peroxidase expression was not tested. The expression of CYP1A1, 1B1 and 3A4 was proved by Western blot analysis (see Fig. 2 for expression of CYP1B1). Whereas no effect of pre-treatment of MCF-7 cells with ellipticine was found for CYP1A1 and 3A4 [data not shown], an increase in CYP1B1 expression was induced by this process. Moreover, levels of this enzyme increased by pre-treatment of MCF-7 cells with a low concentration of ellipticine (0.1 μM) prior to their exposure to 2.5 and 5 μM ellipticine. In addition, a longer period of cell exposition to ellipticine produced higher expression of CYP1B1 (Fig. 2).

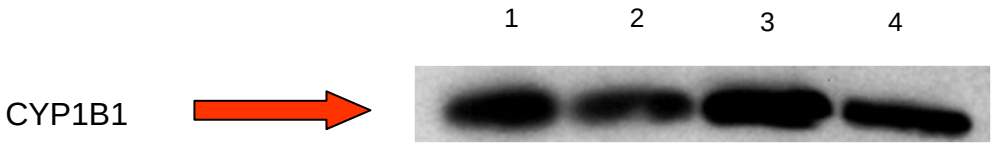

Figure 2. Expression of CYP1B1 in MCF-7 cells. Lane 1, cells pre-treated with 0.1 μM ellipticine for 72 h and then exposed to 2.5 μM ellipticine for 24 h, lane 2, cells exposed to 2.5 μM ellipticine only for 24 h, lane 3, cells pre-treated with 0.1 μM ellipticine for 72 h and then exposed to 5 μM ellipticine for 48 h, lane 4, cells exposed to 5 μM ellipticine only for 48 h.

3.2 Ellipticine-DNA adduct formation in cancer cells

Treatment of tumor cells, in which CYP and/or peroxidase enzymes are expressed, with ellipticine resulted in the formation of the same ellipticine-derived DNA adducts that were formed by enzymatic activation of ellipticine *in vitro* and *in vivo* (see Figure 3 for adducts formed by ellipticine in MCF-7 cells, in rats *in vivo* and in DNA treated with 13-hydroxy- and 12-hydroxyellipticine) [3,4,8,9]. Two major ellipticine-DNA adducts (spots 1 and 2 in Figure 3) that are generated by 13-hydroxy- and

12-hydroxyellipticine (Figs. 3D and 3E), were formed in all tested cells [HL-60 promyelocytic leukemia and T-cell leukemia CCRF-CEM cells, glioblastoma U87MG cells, BHT-101, B-CPAP and 8505-C thyroid cancer cells, neuroblastoma UKF-NB-3 and UKF-NB-4 cell lines (data shown in [20-23,25]) and breast adenocarcinoma MCF-7 cells (Figs. 3A and 3B)] exposed to ellipticine. Moreover, two additional minor adducts (adducts spots 6 and 7 in Figure 3), but the structure of them is not known, are generated in several of tested cancer cells (Table 1). Levels of ellipticine-DNA adducts found in this work and the former studies [20-23,25] are shown in Table 1.

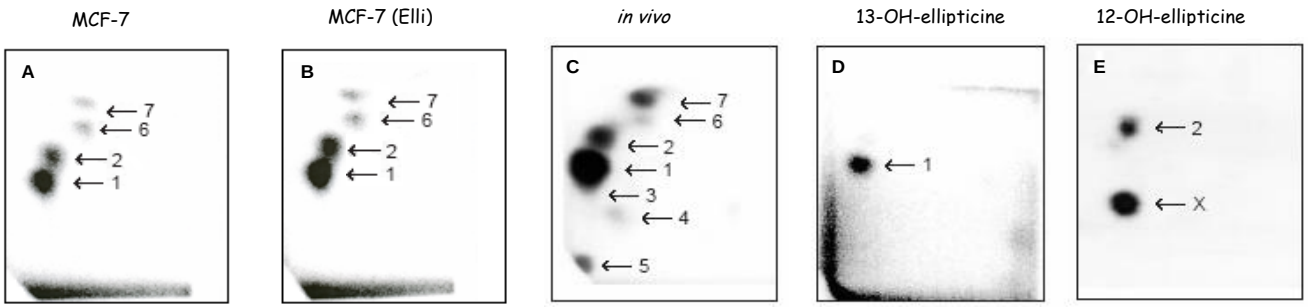

Figure 3. Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA isolated from MCF-7 (A), and from MCF-7 (Elli), the cells pre-treated with 0.1 μM ellipticine for 72 h (B), exposed to 5 μM ellipticine for 24 h, of liver DNA of rats treated with 40 mg ellipticine/kg body weight (C), from calf thymus DNA reacted with 13-hydroxyellipticine (D) and 12-hydroxyellipticine (E). Adduct spots 1-7 correspond to the ellipticine-derived DNA adducts. 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.

3.3. Cytotoxicity of ellipticine to human cancer cells

As shown in our previous studies [20-23,25], the cytotoxicity of ellipticine to tested cancer cell lines, which were treated with the increasing concentrations of ellipticine, were measured with MTT assay. Ellipticine inhibited the growth of all cell lines tested in this and our former studies in a dose-dependent manner [20-23,25]. The IC_{50} values for ellipticine calculated from the dose-log response curves are shown in Table 1. Neuroblastoma UKF-NB-4 and UKF-NB-3 and leukemia HL-60 cells were the most sensitive cells to ellipticine having the IC_{50} values lower than 1 μM (see IC_{50} values shown in Table 1). When we compared the sensitivity of additional cells to ellipticine, similar cytotoxicity of this agent was found to human breast adenocarcinoma MCF-7 cells and a glioblastoma U87MG cell line (the IC_{50} values were app. 1 μM), whereas leukemia CCRF-CEM, and thyroid cancer BHT-101, 8505-C and B-CPAP cells were less sensitive. The IC_{50} values for ellipticine in cancer cells (BHT-101 thyroid cancer cells and leukemia CCRF-CEM cells), which were less sensitive to ellipticine , were 10-times higher than in the most sensitive cells as neuroblastoma UFK-NB-3 and UKF-NB-4 cells (Table 1).

3.4. Induction of CYP1B1 increases ellipticine-DNA adduct formation and cytotoxicity of ellipticine in human breast adenocarcinoma MCF-7 cells

In the present study, the induction of CYP1B1 in human breast adenocarcinoma MCF-7 cells mediated by their pre-treatment with ellipticine was investigated. Pretreatment of these cells with 0.1 μM ellipticine for 72 h resulted in an increase in formation of ellipticine-derived DNA adducts in these cells exposed thereafter to 2.5 and 5 μM ellipticine for 24 h (Table 2).

Table 1. DNA adduct formation by ellipticine and cytotoxicity of this agent in human cancer cell lines

Cells	Levels of DNA adducts (RAL x 10^{-7})[a]					IC_{50} (μM)
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total	
HL-60	46.32 ± 4.30	21.18 ± 2.30	n.d.	n.d.	67.50 ± 6.23	0.64 ± 0.06
CCRF-CEM	9.40 ± 0.95	8.40 ± 0.79	n.d	n.d	17.80 ± 1.62	4.70 ± 0.48
U87MG	1.98 ± 0.15	3.42 ± 0.33	n.d.	n.d.	5.40 ± 0.53	1.48 ± 0.62
UKF-NB-3	3.27 ± 0.32	5.01 ± 0.50	n.d.	n.d.	8.28 ± 0.80	0.44 ± 0.03
UKF-NB-4	5.40 ± 0.56	6.50 ± 0.81	0.27 ± 0.03	0.37 ± 0.05	12.54 ± 1.51	0.49 ± 0.04
BHT-101	3.90 ± 0.28	3.00 ± 0.50	0.10 ± 0.01	0.04 ± 0.01	7.04 ± 0.86	4.80 ± 2.50
B-CPAP	3.33 ± 0.31	4.18 ± 0.42	0.09 ± 0.01	0.03 ± 0.01	7.63 ± 0.76	2.80 ± 1.00
8505-C	2.22 ± 0.56	1.81 ± 0.28	0.05 ± 0.01	0.01 ± 0.01	4.09 ± 0.45	3.60 ± 1.20
MCF-7	3.72 ± 0.40	4.77 ± 0.50	0.81 ± 0.07	n.d.	9.30 ± 0.92	1.25 ± 0.13

Cancer cells were exposed to 10 μM ellipticine for 48 h. DNA adducts were analyzed by the nuclease P1 version of the ^{32}P-postlabeling assay. [a]RAL, relative adduct labeling; averages and S.D. of three experiments. n.d. - not detected (the detection limit of RAL was $1/10^{10}$ nucleotides). IC_{50} values were calculated from the linear regression of the dose-log response curves. Values are mean ± S.D. of at least 3 experiments. Data are taken from [19-23,25].

Table 2. DNA adduct formation by ellipticine and cytotoxicity of this agent in human MCF-7 cells

Cells	Levels of DNA adducts (RAL x 10^{-7})[a]					IC_{50} (μM)
	Adduct 1	Adduct 2	Adduct 6	Adduct 7	Total	
MCF-7						
+ 2.5 μM ellipticine	2.12 ± 0.21	1.38 ± 0.14	0.38 ± 0.04	0.23 ± 0.02	4.11 ± 0.41	1.25 ± 0.13
+ 5 μM ellipticine	3.45 ± 0.35	1.59 ± 0.16	0.56 ± 0.06.	0.45 ± 0.05	6.05 ± 0.61	
MCF -7 (Elli)						
+ 2.5 μM ellipticine	2.95 ± 0.30	1.43 ± 0.14	0.43 ± 0.04	0.23 ± 0.02	5.04 ± 0.50**	0.70 ± 0.07***
+ 5 μM ellipticine	6.37 ± 0.64	2.55 ± 0.26	0.76 ± 0.08	0.64 ± 0.06	10.30 ± 1.10***	

MCF-7 cells and MCF-7 (Elli) , the cells pre-treated with 0.1 μM ellipticine for 72 h, were exposed to 2.5 or 5 μM ellipticine for 24 h. DNA adducts were analyzed by the nuclease P1 version of the ^{32}P-postlabeling assay. [a]RAL, relative adduct labeling; averages and S.D. of three experiments. n.d. - not detected (the detection limit of RAL was $1/10^{10}$ nucleotides). IC_{50} values were calculated from the linear regression of the dose-log response curves. Values are mean ± S.D. of at least 3 experiments. Comparison was performed by *t*-test analysis; $^{**}P < 0.01$, $^{***}P < 0.001$, different from cells that were not pretreated with ellipticine.

In addition, the IC_{50} values for ellipticine in MCF-7 cells indicate that their pretreatment with 0.1 μM ellipticine led to an increase in toxicity of ellipticine to these cells. The IC_{50} value of ellipticine for the control (untreated) and the pretreated cells are 1.25 and 0.7 μM, respectively (Table 2). These results indicate that expression levels of this enzyme are responsible for the increase in formation of DNA adducts by ellipticine and cytotoxicity caused by this drug in these cells. Because no effect of pre-treatment of MCF-7 cells with ellipticine was found for CYP1A1 and 3A4, the CYP1B1 enzyme seems to play a crucial role determining formation of DNA adducts and cytotoxicity of ellipticine in these cells.

4. DISCUSSION

In this study, comparison of cytotoxicity of ellipticine and its relation to expression of the key enzymes catalyzing activation of ellipticine to metabolites forming covalent DNA adducts found in several human cancer cell lines (leukemia, glioblastoma, neuroblastoma, breast adenocarcinoma and thyroid cancer cells) in this and in the previous studies [19-23,25] was carried out. This study also presents the results showing the effects of induction of some of the enzymes metabolizing ellipticine on DNA adduct formation by ellipticine and its cytotoxicity. All these results helped us to shed more light on the mechanism of ellipticine toxic action to these cells.

The mode of antitumor, cytotoxic, mutagenic and genotoxic action of ellipticine is considered to be predominantly based on DNA damage such as intercalation into DNA, inhibition of topoisomerase II, and formation of covalent DNA adducts mediated by CYPs and peroxidases [1-6,13]. The intercalation of ellipticine into DNA and inhibition of topoisomerase II occur in all cell types irrespective of their metabolic capacity because of the general chemical properties of this drug and its affinity to DNA and topoisomerase II protein [5,6]. Hence, ellipticine should affect various cancer cells in a similar way. This is, however, not the case of cancer cells tested in this comparative study. As shown in Table 1 and Figure 4, cytotoxicity of ellipticine (expressed as the IC_{50} values) differs in individual cells and corresponds to levels of ellipticine-derived DNA adducts in most cells. Generally, higher ellipticine-DNA adduct formation resulted in higher cytotoxicity of this drug to most of the tested cancer cells. This finding indicates that covalent modification of DNA by ellipticine plays an important role in toxicity of ellipticine to these cells.

As shown in this work, the toxic effects of ellipticine based on formation of covalent ellipticine-DNA adducts on several cancer cells are dependent on expression of CYP1A1, 1B1, 3A4 and peroxidases COX-1 and MPO in these cells. This is clearly seen in two leukemia cells tested, HL-60 and CCRF-CEM cells; high expression of one of the enzyme activating ellipticine, MPO, in HL-60 cells and the absence of this peroxidase in a CCRF-CEM cell line [20], resulted in quite different levels of ellipticine-derived DNA adducts and subsequently in ellipticine cytotoxicity in these cells. In addition, not only the basal levels of enzymes metabolizing ellipticine, but the induction effect of ellipticine on expression of several enzymes catalyzing its metabolism might be another factor determining DNA adduct formation by ellipticine and its cytotoxicity in several of the tested cancer cells. Indeed, in human UKF-NB-3 and UKF-NB-4 neuroblastoma cells [21,22], glioblastoma U87MG

[23] cells and adenocarcinoma MCF-7 cells (this work) that are very sensitive to ellipticine, CYP1A1, 3A4 and/or CYP1B1 were, depending on individual cells, induced by ellipticine. The CYP1A1 enzyme was also partially induced in B-CPAP and 8505-C thyroid cancer cell lines and CYP3A4 in BHT-101 and B-CPAP cells [25]. In contrast, exposure of cancer cells to ellipticine had either no (U87MG cells) or inhibition effects (UKF-NB-3, UKF-NB-4, and thyroid cancer cell lines) on expression of peroxidases COX-1 and/or LPO [23,25].

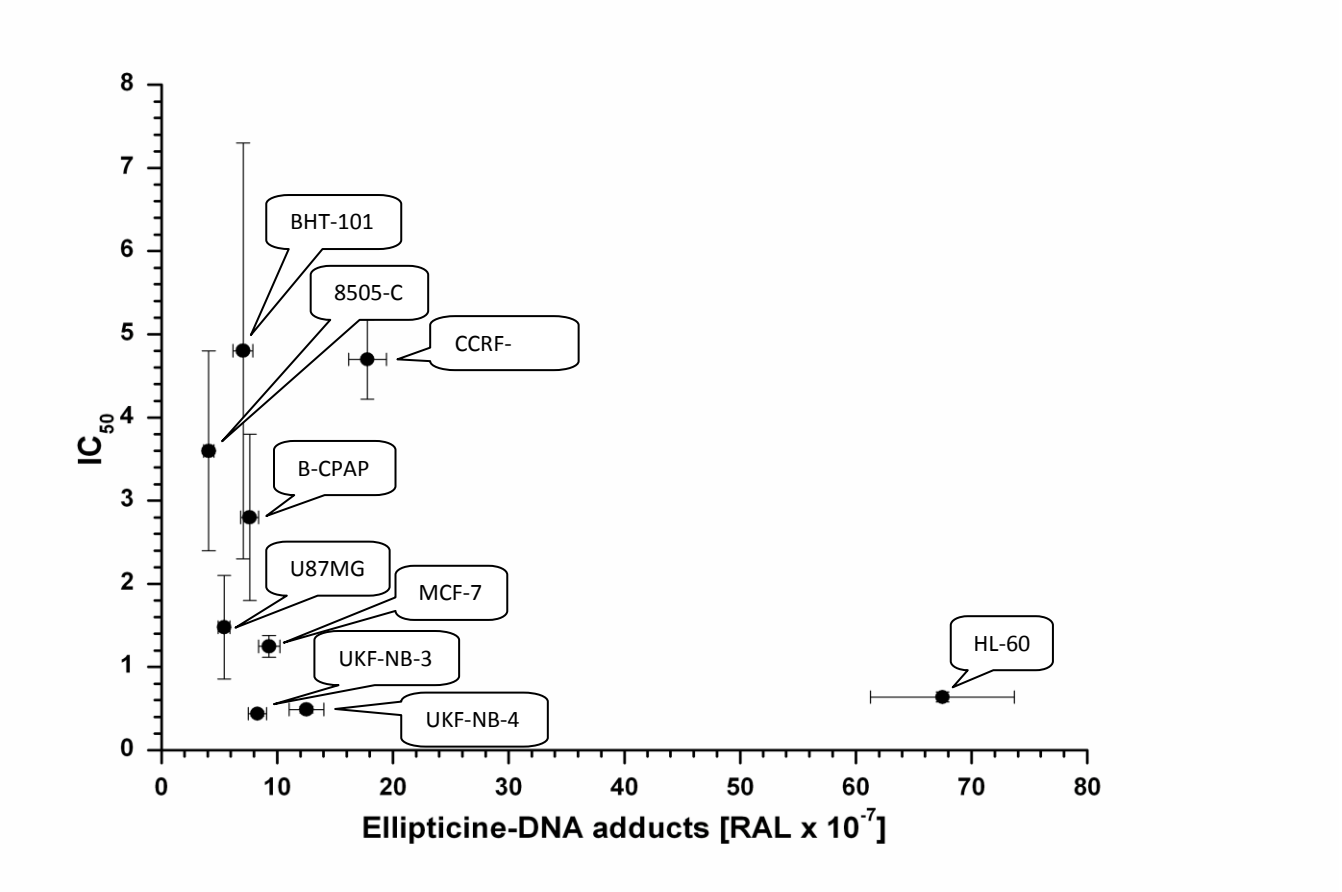

Figure 4. Relationship between the levels of ellipticine-derived DNA adducts and the IC_{50} values for ellipticine in cancer cell lines.

The important effect of induction of enzymes metabolizing ellipticine by this drug on ellipticine cytotoxicity was unambiguously proved in this study, in MCF-7 cells, in which the CYP1B1 was induced by their pre-treatment with low concentrations of ellipticine (0.1 μM). Cytotoxicity of ellipticine in MCF-7, in which CYP1B1 expression was induced, was increased and correlated with higher levels of elllipticine-DNA adducts. Thus, these results indicate that the induction potency of ellipticine exerts concerted regulatory control of this drug on its own metabolic fate and pharmacological efficiency.

The results showed in the present comparative study suggest that ellipticine toxicity to most of the cancer cells tested in this work is dictated by formation of covalent ellipticine-DNA adducts mediated by enzymes catalyzing ellipticine activation in these cells. Nevertheless, this feature is not

absolute. This is, for example, not the case of the UKF-NB-3 cell line; lower levels of DNA adducts were found in these cells than in UKF-NB-4 cells, even though both neuroblastoma cells exhibit similar sensitivity to ellipticine. One of the reasons for this phenomenon might be different genetic programs of both neuroblastoma cells [33,34]. The UKF-NB-4 line was, namely, established from a recurrent disease. In addition, the mechanisms such as intercalation into DNA, inhibition of DNA topoisomerase II activity (for a review see [3-6,13]) or DNA damage caused by reactive oxygen species (ROS) generated during ellipticine oxidation [35,36], might also contribute to ellipticine cytotoxicity in UKF-NB-3 cells. Indeed, recently using two electrochemical methods, square wave voltametry [37,38] and capillary electrophoresis with laser-induced fluorescence [39], ellipticine as an DNA intercalator was found that might, to some extent, also participate in cytotoxicity of this drug in neuroblastoma UKF-NB-3 cells. Nevertheless, an actual contribution of DNA-damaging effects different from the covalent DNA modification by ellipticine, to ellipticine toxicity to cancer cells awaits further investigation.

5. CONCLUSIONS

The results of the present comparative study indicate that levels of protein expression of CYP1A1, 1B1, 3A4, lactoperoxidase, thyroid peroxidase and cyclooxygenase-1 enzymes that catalyze ellipticine metabolism in several cancer cells influence formation of covalent ellipticine-derived DNA adducts and sensitivity of these cells to ellipticine. The results found also demonstrate a potency of ellipticine to induce the enzymes dictating ellipticine activation to metabolites forming DNA adducts and this induction can lead to an increase in cytotoxicity of ellipticine in most studied cancer cells.

ACKNOWLEDGEMENTS
Financial support from GACR (grant P301/10/0356) and Charles University in Prague (UNCE 204025/2012) is highly acknowledged.

References

1. M. Stiborova, C.A. Bieler, M. Wiessler and E. Frei, *Biochem. Pharmacol.*, 62 (2001) 1675.
2. M. Stiborova, M. Rupertova, H. H. Schmeiser and E. Frei, *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.*, 150 (2006) 13.
3. M. Stiborova, M. Rupertova and E. Frei, *Biochim. Biophys. Acta*, 1814 (2011) 175.
4. R. Kizek, V. Adam, J. Hrabeta, T. Eckschlager, S. Smutny, J. V. Burda, E. Frei and M. Stiborova, *Pharmacol. Ther.*, 133 (2012) 26.
5. C. Auclair, *Arch. Biochem. Biophys.*, 259 (1987) 1.
6. N. C. Garbett and D. E. Graves, *Curr. Med. Chem. Anti-Cancer Agents*, 4 (2004) 149.
7. K. Tmejova, L. Krejcova, D. Hynek, V. Adam, P. Babula, L. Trnkova, M. Stiborova, T. Eckschlager and R. Kizek, *Anti-Cancer Age. Med.*, 14 (2014), 331.
8. M. Stiborova, J. Sejbal, L. Borek-Dohalska, D. Aimova, J. Poljakova, K. Forsterova, M. Rupertova, J. Wiesner, J. Hudecek, M. Wiessler and E. Frei, *Cancer Res.*, 64 (2004) 8374.
9. M. Stiborova, J. Poljakova, H. Ryslava, M. Dracinsky, T. Eckschlager and E. Frei, *Int. J. Cancer*, 120 (2007) 243.

10. M. Stiborova, M. Rupertova, D. Aimova, H. Ryslava and E. Frei, *Toxicology*, 236 (2007) 50.
11. M. Stiborova, R. Indra, M. Moserova, V. Cerna, M. Rupertova, V. Martinek, T. Eckschlager, R. Kizek and E. Frei, *Chem. Res. Toxicol.*, 25 (2012) 1075.
12. V. Kotrbova, B. Mrazova, M. Moserova, V. Martinek, P. Hodek, J. Hudecek, E. Frei and M. Stiborova, *Biochem. Pharmacol.*, 82 (2011) 669.
13. M. Stiborova and E. Frei, *Current Med. Chem.*, 21 (2014) 575.
14. M. Moserova, V. Kotrbova, M. Rupertova, K. Naiman, J. Hudecek, P. Hodek, E. Frei and M Stiborova, *Neuro Endocrinol. Lett.*, 29 (2008) 728.
15. M. Stiborova, A. Breuer, D. Aimova, M. Stiborova-Rupertova, M. Wiessler and E. Frei, *Int J Cancer* 107 (2003) 885.
16. M. Stiborova, M. Rupertova, D. Aimova, H. Ryslava and E. Frei, *Toxicology*, 236 (2007) 50.
17. M. Stiborova, V.M. Arlt, C.J. Henderson, C.R. Wolf, V. Kotrbova, M. Moserova, J. Hudecek, D.H. Philips and E. Frei, *Toxicol. Appl. Pharmacol.*, 226 (2008) 318.
18. M. Stiborova, M. Moserova, B. Mrazova, V. Kotrbova and E. Frei, *Neuro Endocrinol. Lett.*, 31 (Suppl. 2) (2010) 26.
19. L. Borek-Dohalska, E. Frei and M. Stiborová, *Collect. Czech. Chem. Commun.*, 69 (2004) 603.
20. J. Poljakova, E. Frei, J.E. Gomez, D. Aimova, T. Eckschlager, J. Hrabeta and M. Stiborova, *Cancer Lett.*, 252 (2007) 270.
21. J. Poljakova, T. Eckschlager, J. Hrabeta, J. Hrebackova, S. Smutny, E. Frei E, V. Martínek, R. Kizek and M.Stiborová, *Biochem Pharmacol.*, **77** (2009) 1466.
22. J. Poljakova, J. Hrebackova, M. Dvorakova, M. Moserova, T. Eckschlager, J. Hrabeta, M. Göttlicherova, B. Kopejtkova, E. Frei, R. Kizek and M. Stiborova, *Neuro Endocrinol. Lett.*, 32, Suppl. 1 (2011) 101.
23. E. Martinkova, M. Dontenwill, E. Frei and M. Stiborova, *Neuro Endocrinol. Lett.*, 30, Suppl. 1 (2009) 60.
24. L. Bořek-Dohalská., J. Poljaková, M. Rupertová, E. Frei and M. Stiborová, *Interdisc. Toxicol.*, 2 (2009), 94.
25. J. Poljakova, T. Eckschlager, R. Kizek, E. Frei and M. Stiborová, *Int. J. Electrochem. Sci.*, 8 (2013) 1573.
26. M. Stiborova, V. Martinek, H. Rydlova, P. Hodek and E. Frei, *Cancer Res.*, 62 (2002) 5678.
27. M. Stiborova, V. Martinek, H. Rydlova, T. Koblas and P. Hodek, *Cancer Lett*. 220 (2005) 145.
28. D. Aimova, L. Svobodova, V. Kotrbova, B. Mrazova, P. Hodek, J. Hudecek, R. Vaclavikova, E. Frei and M. Stiborova, *Drug Metab. Dispos.* 35 (2007) 1926.
29. I. Vranová, M. Moserová, P. Hodek, R. Kizek, E. Frei and M Stiborová, *Int. J. Electrochem. Sci.*, 8 (2013) 1586.
30. P. Hodek, P. Trefil, J. Šimůnek, J. Hudeček and M Stiborova, *Int. J. Electrochem. Sci.*, 8 (2013) 113.
31. E. Frei, C.A. Bieler, V.M. Arlt, M. Wiessler and M Stiborová, *Biochem. Pharmacol.*, 64 (2002) 289.
32. M. Stiborová, J. Poljakova, E. Martínková, J. Ulrichová, V. Šimánek, Z. Dvořák and E. Frei, *Toxicology*, 302 (2012), 233.
33. J. Cinatl Jr, J. Cinatl, R. Kotchetkov, J.U. Vogel, B.G. Woodcock,, J. Matousek J, P. Pouckova and B. Kornhuber, *Int. J. Oncol* ., 15 (1999) 1001.
34. J. Bedrnicek, A. Vicha, M. Jarosova, M. Holzerova, J. Cinatl Jr, M. Michaelis, J. Cinatl and T. Ecschlager, *Neoplasma* 52: (2005) 415.
35. J.Y. Kim, S.G. Lee, J.Y. Chung, Y.J. Kim, J.E. Park, H. Koh, M.S. Han, Y.C. Park, Y.H. Yoo and J.M. Kim, *Toxicology,* 289 (2011) 91.
36. M. Pohanka. J. Sochor, B. Ruttkay-Nedecký, N. Cerii, V. Adam, J. Hubálek, M. Stiborová, T. Eckschlager and R. Kízek, *J. Appl. Biomed.*, 10 (2012) 155.

37. D. Huska, V. Adam,, S. Krizkova, J. Hrabeta, T. Eckschlager, M. Stiborova and R. Kizek, *Chim. Oggi-Chem. Today*, 28 (5, Suppl. S) (2010) 15.
38. D. Huska, V. Adam, J. Hubalek, L. Trnkova,, T. Eckschlager, M. Stiborova, I. Provaznik and R. Kizek, *Chim. Oggi-Chem. Today*, 28 (5, Suppl. S) (2010) 18.
39. M. Ryvolova, J. Chomoucka, J. Drbohlavova, P. Kopel, P. Babula, H. Hynek, V. Adam, T. Eckschlager, J. Hubalek, M. Stiborova, J. Kaiser and R. Kizek, *Sensors*, 12 (2012) 14792.