

Analysis of covalent ellipticine- and doxorubicin-derived adducts in DNA of neuroblastoma cells by the ^{32}P -postlabeling technique

This work is dedicated to Professor Gustav Entlicher on the occasion of his 70th birthday

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Background. Ellipticine and doxorubicin are antineoplastic agents, whose action is based mainly on DNA damage such as intercalation, inhibition of topoisomerase II and formation of covalent DNA adducts. The key target to resolve which of these mechanisms are responsible for ellipticine and doxorubicin anticancer effects is the development of suitable methods for identifying their individual DNA-damaging effects. Here, the ^{32}P -postlabeling method was tested to detect covalent DNA adducts formed by ellipticine and doxorubicin.

Methods. The standard procedure of ^{32}P -postlabeling assay, this procedure under ATP-deficient conditions, the version using extraction of adducts with *n*-butanol and the nuclease P1 enrichment version were used to analyze ellipticine- and/or doxorubicin-derived DNA adducts.

Results. Two covalent ellipticine-derived DNA adducts, which are associated with cytotoxicity of ellipticine to human UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines, were detected by the ^{32}P -postlabeling method. These adducts are identical to those formed by the ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine. In contrast, no covalent adducts formed by doxorubicin in DNA of these neuroblastoma cells and in DNA incubated with this drug and formaldehyde *in vitro* were detectable by the ^{32}P -postlabeling assay.

Conclusions. The results presented in this paper are the first to demonstrate that in contrast to covalent DNA adducts formed by ellipticine, the adducts generated by formaldehyde-mediated covalent binding of doxorubicin to DNA are not detectable by the ^{32}P -postlabeling assay. No DNA adducts were, detectable either *in vitro*, in incubations of DNA with doxorubicin or in DNA of neuroblastoma cells treated with this drug. The results also suggest that covalent binding of ellipticine to DNA of UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines is the predominant mechanism responsible for the cytotoxicity of this drug. To understand the mechanisms of doxorubicin anticancer effects on neuroblastoma cells, development of novel methods for identifying covalent doxorubicin-derived DNA adducts is the major challenge for further research.

Key words: ellipticine, doxorubicin, neuroblastoma, DNA adducts, ^{32}P -postlabeling, cancer

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INTRODUCTION

Neuroblastoma, a tumor of the peripheral sympathetic nervous system, is the most frequent solid extracranial neurological tumor in children and is a major cause of death from neoplasia in infancy^{1,2}. These tumors are biologically heterogeneous, with cell populations differing in their genetic programs, maturation stage and malignant potential³. Neuroblastoma consists of three principal neoplastic cells^{4,5}: i) neuroblastic or N-type: undifferentiated, round and small cells with scant cytoplasm and neuritic

processes; ii) stromal or S-type: large, flattened and adherent differentiated cells; and iii) intermediate or I-type with morphological features of both above mentioned types i.e. cells with short neurite-like processes such as adherent growth. As neuroblastoma cells seem to have the capacity to differentiate spontaneously *in vivo* and *in vitro*⁶, their heterogeneity could affect treatment outcome, in particular the response to apoptosis induced by chemotherapy. Neuroblastoma may regress spontaneously in infants, mature to benign ganglioneuromas, or grow relentlessly and be rapidly fatal³. The prognosis for patients with high

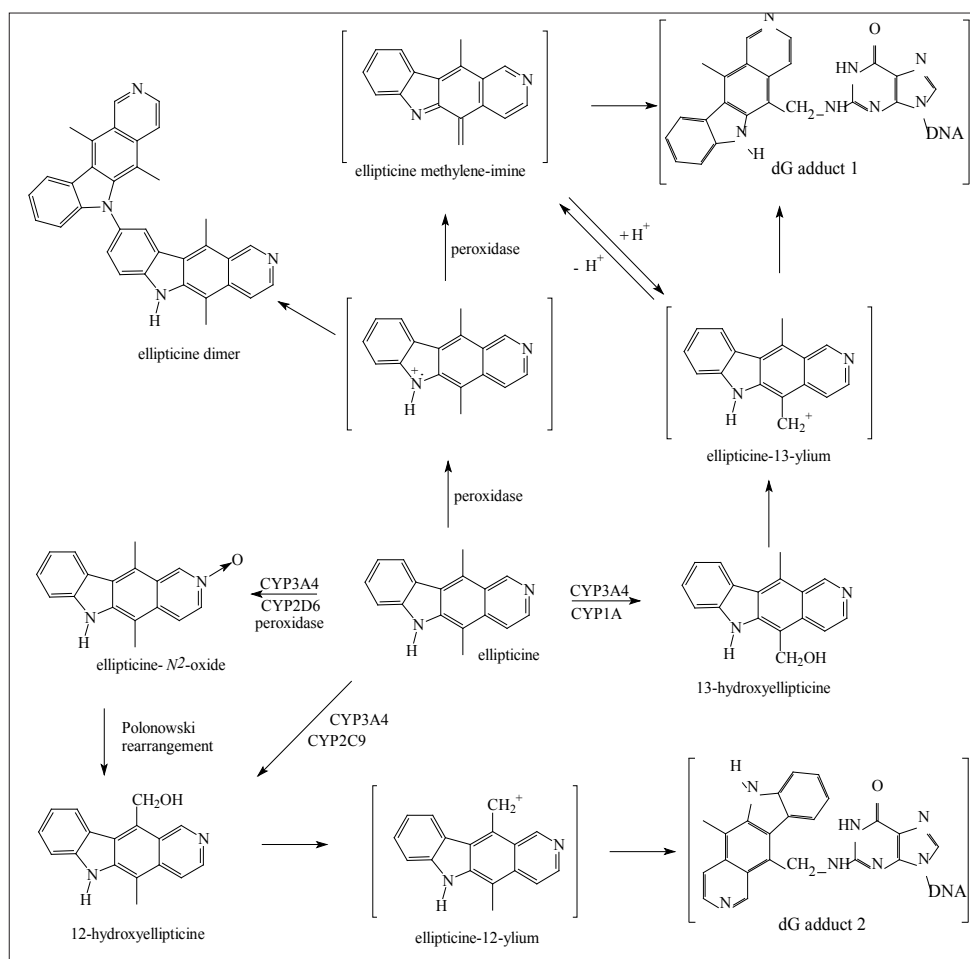


Fig. 1. Scheme of the metabolism of ellipticine by human CYPs and peroxidases showing the characterized detoxication metabolites and those proposed to form DNA adducts. The compounds shown in brackets are the hypothetical electrophilic metabolites postulated as ultimate arylating species or the postulated *N*²-deoxyguanosine adducts.

risk tumors is poor, in spite of intensive therapy including megatherapy with subsequent hematopoietic progenitor cell transplantation, biotherapy and immunotherapy because drug resistance arises in the majority of these patients who initially responded to chemotherapy³.

Chemotherapy agents used in combination have been found to be effective against neuroblastoma. Agents commonly used are platinum compounds (carboplatin), alkylating agents (cyclophosphamide, ifosfamide, melphalan), topoisomerase II inhibitors (etoposide), anthracycline antibiotics (doxorubicin) and vinca alkaloids (vincristine). Some novel regimens include also topoisomerase I inhibitors (topotecan and irinotecan), which have been found to be effective against recurrent disease³. Nevertheless, little improvement in therapeutic options has been made in the last decade, requiring a need for the development of new therapies.

Recently, we have suggested novel treatment of neuroblastomas, utilizing a drug targeting DNA, the plant alkaloid ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig.1). We found that exposure of human neuroblastoma IMR-32, UKF-NB-3 and UKF-NB-4 cell lines to this agent resulted in strong inhibition of cell growth, followed by induction of apoptosis⁷. These effects were associated with formation of two covalent ellipticine-derived DNA adducts, identical to those formed by the cytochrome P450 (CYP)- and peroxidase-mediated ellipticine metabolites,

13-hydroxy- and 12-hydroxyellipticine (Fig. 1). In addition, other mechanisms, such as intercalation into DNA (ref.⁸), and inhibition of DNA topoisomerase II activity⁸⁻¹¹ resulting in ellipticine toxicity to neuroblastoma cannot be excluded (for a summary see¹²⁻¹⁵).

Of anthracycline antibiotics, doxorubicin (Fig. 2A) is frequently used for neuroblastoma treatment³. The primary mechanism of action of doxorubicin (and other anthracyclines) appears to be poisoning of the enzyme topoisomerase II which results in double-strand DNA breaks, and the failure to repair these breaks leads to apoptosis^{15,16}. More recently however, it has been demonstrated that doxorubicin also forms covalent adducts with DNA and these lesions are more cytotoxic than those induced by topoisomerase II impairment¹⁷. The adducts are formed predominantly at 50-GC-30 sites in DNA (ref.¹⁸), where the doxorubicin sugar group (daunosamine) is covalently linked to the N-2 amino group of guanine via an aminal (N-C-N) bond^{19,22}. The central carbon atom in the aminal bond is derived from formaldehyde, hence formaldehyde is an absolute requirement for adduct formation^{20,21}. This compound is present in cancer cells, at levels which are often even higher in tumor cells (1.5-4.0 mM) than normal cells^{23,24}. Moreover, formaldehyde is also produced in cancer cells treated with doxorubicin, from the oxidation of doxorubicin itself²⁰. The resulting drug-DNA monoadduct is further stabilized through in-

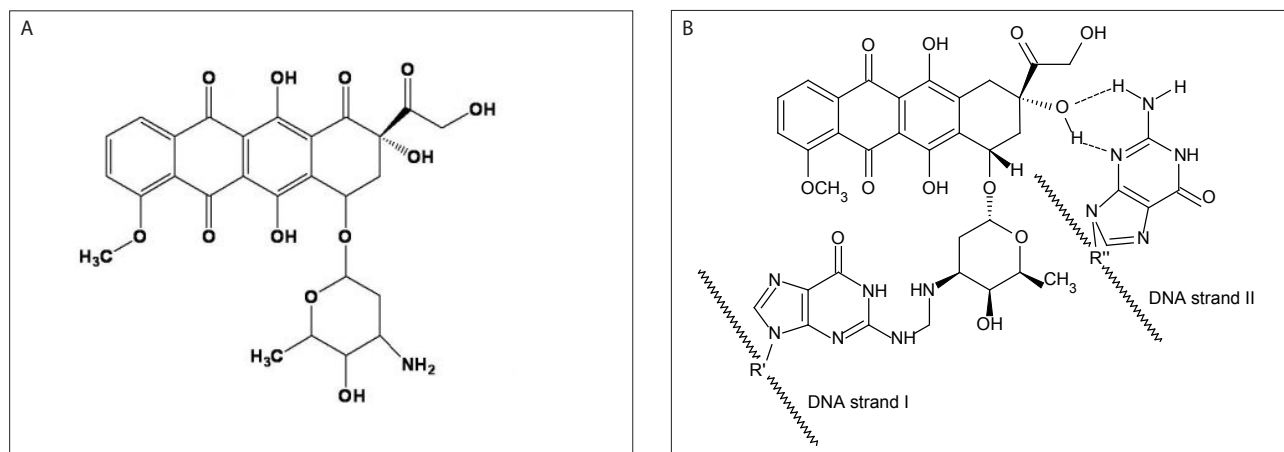


Fig. 2. Structures of doxorubicin (A) and doxorubicin-DNA adduct (B) (adapted from²⁷).

tercalation and hydrogen bonding with the second strand of DNA (ref.²¹) (Fig. 2B).

Even though ellipticine and doxorubicin are cytotoxic to neuroblastomas⁷, the question arises whether the covalent DNA adduct formation is the predominant mechanism responsible for this cytotoxic effect. To answer this question, development of methods suitable for detecting and quantifying covalent DNA adducts formed by ellipticine and doxorubicin is the first and key target of the research. Using ¹⁴C-labeled doxorubicin, doxorubicin-DNA adduct formation has been detected in several cancer cells^{17,20,21,25}. Moreover, accelerator mass spectrometry has recently also been shown suitable for detecting the covalent ¹⁴C-labeled doxorubicin-DNA adducts in cancer cells exposed to this drug²⁶⁻²⁸. Utilizing the ¹⁴C-labeled doxorubicin has, however, limitations for use in human treatment.

³²P-postlabeling is the commonest method for determination of DNA adducts formed by several substances that covalently modify DNA (ref.²⁹⁻³²). However, whereas it is useful for detecting and quantifying covalent ellipticine-derived DNA adducts^{12,14,15,33,34}, its ability to determine covalent doxorubicin-DNA adducts remains to be resolved. The present study was therefore undertaken to investigate whether covalent doxorubicin-DNA adducts are detectable by the ³²P-postlabeling technique. DNA adducts formed by doxorubicin in DNA of neuroblastoma cells and in DNA incubated with this drug and formaldehyde were analyzed. Since neuroblastoma is heterogenous and this feature could affect its treatment, two types of neuroblastoma cell lines were tested for their response to treatment by ellipticine and doxorubicin, UKF-NB-3 cells (the invasive N-type), and UKF-NB-4 cells (the less-aggressive S-type). DNA from UKF-NB-3 and UKF-NB-4 cell lines treated with ellipticine and doxorubicin in concentrations that are toxic to these cells was isolated and formation of covalent DNA adducts by ellipticine and doxorubicin analyzed.

MATERIAL AND METHODS

Chemical

Ellipticine was obtained from Sigma (St. Louis, MO, USA). Doxorubicin was obtained from EBEWE Pharma Ges.m.b.H. (Unterach, Austria), dimethyl sulfoxide (DMSO) from Amresco Inc. (Solon, OH, USA), phenolchloroform from Roth (Karlsruhe, Germany) and isopropanol from PLIVA-Lachema (Brno, Czech Republic). 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^{12,33,34}. All these and other chemicals used in the experiments were of analytical purity or better. Enzymes and chemicals for the ³²P-postlabeling assay were obtained from sources described¹². All other chemicals used in the experiments were of analytical purity or better.

Synthesis of covalent formaldehyde-mediated doxorubicin-DNA adducts

Multiple 0.5-ml reactions were run in parallel according the procedure described by Zeman et al.²¹. Briefly, each reaction mixture contained 125 mM calf thymus DNA, 125 mM doxorubicin, and 0.37% formaldehyde in 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl and 0.5 mM EDTA. Reactions were run at 10 °C for 4 h, the time determined for nearly 100% conversion of all DNA to covalent species²¹. DNA was isolated from the mixture by its precipitation with 2.5 times the volume of ethanol (-20 °C), washed twice with 5 ml of 70% ethanol, 5 ml of absolute ethanol and 5 ml of diethyl ether. DNA was dried under a stream of nitrogen and dissolved in distilled water.

Cell cultures

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). UKF-NB-4 was derived from recurrent disease. Both cell lines used were derived from high risk neuroblastoma with MYCN am-

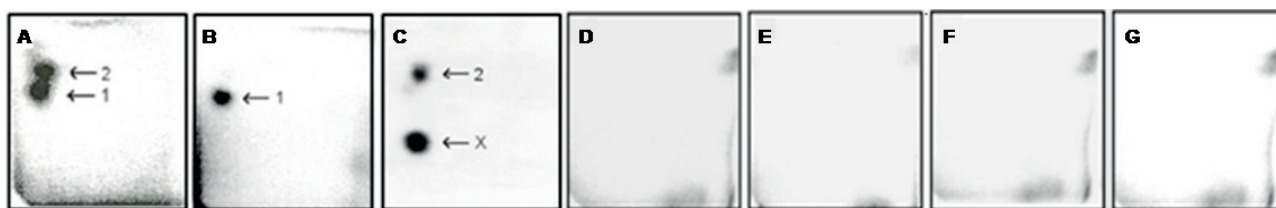


Fig. 3. Autoradiographs of PEI-cellulose TLC maps of ^{32}P -labeled digests of DNA isolated from neuroblastoma UKF-NB-3 exposed to 10 μM ellipticine for 48 h (A) (ref.⁷), from calf thymus DNA reacted with 13-hydroxyellipticine (B) and 12-hydroxyellipticine (C) (ref.^{33,34}). Analyses were performed by the nuclease P1 version of the ^{32}P -postlabeling assay. (D-G) Autoradiographs of PEI-cellulose TLC maps of ^{32}P -labeled digests of DNA isolated from neuroblastoma UKF-NB-3 exposed to 10 μM doxorubicin for 48 h. The standard procedure of the ^{32}P -postlabeling assay was used for the TLC map in panel (D), the standard procedure under ATP-deficient conditions for that in panel (E), the nuclease P1 version for that in panel (F) and the version utilizing extraction of adducts into *n*-butanol for that in panel (G). The method A (see the Material and methods section) was utilized for resolution of adducts in all panels. (A) Scans of the plates from the imager for 6.5 min; (B,C) autoradiographs of films exposed for 1 h at -80°C ; (D-G) scans of the plates from the imager for 1 h. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

plification, del1p and aneuploidy. Cells were grown at 37°C and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (Lonza Inc, Allendale, NJ, USA), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml of penicilline and 100 $\mu\text{g}/\text{ml}$ streptomycine (PAA Laboratories, Pasching, Austria). For hypoxia experiments, cells were maintained in modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) flushed with 1% O_2 , 5% CO_2 and balance N_2 for 4 min (hereafter referred to as hypoxia). This chamber was placed into 37°C .

Treatment of neuroblastoma cells with ellipticine and doxorubicin for DNA adduct analyses

Neuroblastoma cell lines were seeded 24 h prior to treatment at a density of 5×10^5 cells/ml in two 75 cm^2 culture flasks in a total volume of 20 ml of IMDM. Ellipticine and doxorubicin were dissolved in 5 μl of DMSO and distilled water, respectively. The final concentration was 1 or 10 μM . After 48 h the cells were harvested after trypsinizing by centrifugation at $2000 \times g$ for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at -80°C until DNA isolation. DNA was isolated and labeled as described in the next section.

DNA isolation

DNA from cells treated with ellipticine and doxorubicin was isolated by the phenol-chloroform extraction as described⁷.

^{32}P -postlabeling of ellipticine-derived DNA adducts

The ^{32}P -postlabeling of nucleotides using nuclease P1 enrichment procedure³¹, found previously to be appropriate to detect and quantify ellipticine-derived DNA adducts formed *in vitro* and *in vivo*^{12,14,33,34} was used previously to detect and quantify the adducts formed in neuroblastoma cells⁷. DNA isolated from neuroblastoma cells as described in that study⁷ and DNA from experiments performed earlier, namely, calf thymus DNA incubated with 13-hydroxy- and 12-hydroxyellipticine^{33,34} were labeled with ^{32}P to show and compare adduct spot patterns.

^{32}P -postlabeling of doxorubicin-derived DNA adducts

The standard procedure²⁹, this procedure under the ATP-deficient conditions³⁰, the version using extraction of adducts with *n*-butanol³⁵ and the nuclease P1 enrichment version³¹ of the ^{32}P -postlabeling assay were used. Labeled DNA digests were separated by two chromatographic methods on polyethylenimine (PEI)-cellulose plates. (i) Essentially as described²⁹, except that D3 solvent was 3.5 M lithium formate, 8.5 M urea (pH 3.5); D4 solvent was 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea (pH 8.0), followed by a final wash with 1.7 M sodium phosphate (pH 6.0). D2 was omitted (method A). (ii) ^{32}P -labeled adducts were also resolved by a modification described by Reddy et al³². This procedure has been shown to be suitable for resolution of DNA adducts formed by *o*-anisidine³⁶ or *o*-nitroanisole³⁷. The solvents used in this case were: D1, 2.3 M sodium phosphate (pH 5.77); D2 was omitted; D3, 2.7 M lithium formate, 5.1 M urea (pH 3.5); D4, 0.36 M sodium phosphate, 0.23 M Tris-HCl, 3.8 M urea (pH 8.0). After D4 development and brief water wash, the sheets were developed (along D4) in 1.7 M sodium phosphate (pH 6.0) (D5), to the top of the plate, followed by an additional 30-40 min development with the TLC tank partially opened, to allow the radioactive impurities to concentrate in a band close to the top edge (method B)(ref.^{32,36,37}). Adduct levels were calculated in units of relative adduct labeling (RAL), which is the ratio of c.p.m. of adducted nucleotides to c.p.m. of total nucleotides in the assay.

RESULTS AND DISCUSSION

DNA adduct formation by ellipticine and doxorubicin analyzed with ^{32}P -postlabeling

As shown in our previous study⁷, ellipticine and doxorubicin are cytotoxic to human UKF-NB-3 and UKN-NB-4 neuroblastoma cells. The toxicity of ellipticine and doxorubicin to both neuroblastoma cell lines was similar; IC_{50} values ranged from 0.42 to 0.70 μM (ref.⁷). It should be noted, however, that even though the IC_{50} values for

ellipticine for both neuroblastoma cell lines did not differ significantly, the UKF-NB-4 cell line (the less-aggressive S-type line) was less sensitive to this drug than the UKF-NB-3 cell line. No viability of a UKF-NB-3 neuroblastoma cell line was found at 0.75 μM ellipticine in cultivation medium, while viability of UKF-NB-4 cells was found up to ellipticine concentrations of 6.3 μM .

DNA adducts were analyzed in DNA of neuroblastoma cells treated with 1 and 10 μM ellipticine and doxorubicin, the concentrations that are toxic to these cells. Formation of ellipticine-derived DNA adducts in UKF-NB-3 and UKN-NB-4 neuroblastoma cells has already been found in our previous work⁷. Therefore, their analysis in human UKF-NB-3 cells was used in the present study as positive control. As shown in Fig. 3A, two major ellipticine-derived DNA adducts formed in these neuroblastoma cells are generated from ellipticine-13-ylidium and ellipticine-12-ylidium (Fig. 1), the reactive species formed by dissociation of ellipticine metabolites, 13-hydroxy- and 12-hydroxyellipticine^{33,34} (Fig. 3B,C). Since ellipticine-derived DNA adducts have already been detected and quantitated using the nuclease P1 version of ³²P-postlabeling assay^{12,33,34}, this version of the method was first used also for analysis of DNA isolated from neuroblastoma cells treated with doxorubicin.

In contrast to these results, no adducts were found in DNA of cells treated with doxorubicin analyzed with the same version of the ³²P-postlabeling method (Fig. 3F). Because the nuclease P1 version of the ³²P-postlabeling method might have limitations for detecting some of DNA adducts³⁵, we also used other versions of the ³²P-postlabeling method such as the standard procedure²⁸, this procedure under the ATP-deficient conditions³⁰ and the version utilizing of extraction of adducts into *n*-butanol³⁵ to analyze doxorubicin-derived DNA adducts (Fig. 3D-G). Likewise, modifications of all versions that are appropriate for resolution of more polar adducts on thin layer of PEI cellulose such as the adducts generated in DNA by *o*-anisidine³⁶ or *o*-nitroanisole³⁷ (see method B in Material and Methods), were tested to determine doxorubicin-derived DNA adducts. Using all these methods, no adducts were again detected (data not shown). These findings might indicate at least two phenomena. First, low levels of covalent adducts (if any) that are undetectable by the ³²P-postlabeling methods might be formed by doxorubicin in DNA of neuroblastoma cells. Their formation in neuroblastoma cells cannot, however, be excluded. Recently, changes in structure of DNA isolated from neuroblastoma cells induced by their treating with doxorubicin have been detected by square-wave voltammetry³⁸. Nevertheless, whether these changes are produced by formation of actual covalent doxorubicin-derived DNA adducts remain to be investigated. Second, the ³²P-postlabeling method is not suitable to determine covalent adducts formed in DNA by doxorubicin.

In order to resolve whether the ³²P-postlabeling method is suitable for detection of covalent formaldehyde-mediated doxorubicin-DNA adduct, we prepared the DNA adduct synthetically, by incubation of DNA with

doxorubicin in the presence of formaldehyde, the compound necessary to covalent doxorubicin-DNA adduct formation²¹. DNA incubated with doxorubicin and formaldehyde was isolated from the incubation mixture (see Material and Methods) and analyzed with all versions of the ³²P-postlabeling method and two modifications used for resolution of adducts on PEI-cellulose TLC (see Materials and Methods). No DNA adducts were again detected (data not shown). These results indicate that none of the versions of the ³²P-postlabeling assay is suitable for detecting formaldehyde-mediated covalent doxorubicin-DNA adducts. We can only speculate why formaldehyde-mediated covalent doxorubicin-DNA adduct is not detectable by ³²P-postlabeling. One of the reasons for this finding, could be inefficient labeling reaction for formaldehyde-mediated doxorubicin-derived adduct during the ³²P-postlabeling method, which is the conversion of the adducted nucleoside 3'-phosphate to its corresponding 3',5'-bisphosphate by T4 polynucleotide kinase. Further reasons could be incomplete digestion of modified DNA, loss of material during the experimental manipulations used in ³²P-postlabeling or retaining compounds at the origin of the PEI-cellulose TLC plates. However, investigation of these features was beyond the scope of the present study.

Cytotoxicity of and DNA adduct formation by ellipticine and doxorubicin in neuroblastoma cells cultivated under hypoxic conditions

Hypoxia frequently occurs in tumors because of their fast growth and inadequate vascularisation. This strongly correlates with advanced disease and poor outcome caused by chemoresistance. As reported⁷, growth inhibition is mediated by ellipticine and doxorubicin in neuroblastoma cells even under hypoxic conditions of cultivation. Nevertheless, whereas a low effect of hypoxia was found on toxicity of both tested compounds to a UKF-NB-3 cell line, the UKF-NB-4 neuroblastoma cells were less sensitive to both studied cytostatics under the hypoxic conditions of their cultivation⁷.

Under hypoxic conditions, the ellipticine-DNA adduct levels in neuroblastoma cells were lower⁷, whereas formation of DNA adducts generated by doxorubicin in these cells was again not detectable. In both neuroblastoma cell lines treated with 10 μM ellipticine, almost 2-fold decrease in levels of ellipticine-DNA adducts by hypoxia was found and resulted from a decreased formation of adducts 1 and 2 in both types of cells⁷. This finding shows that CYP enzymes, whose activities are dependent on oxygen^{13,14,33,34}, are predominantly responsible for formation of adducts 1 and 2 in these neuroblastoma cells⁷.

A decrease in the levels of adducts 1 and 2 in neuroblastoma cells under hypoxic conditions corresponded to a decrease in toxicity of ellipticine under these conditions⁷. We therefore concluded that formation of ellipticine-DNA adducts was the predominant DNA-damaging mechanism of ellipticine action, resulting in its strong cytotoxicity to neuroblastoma cells.

CONCLUSIONS

The results presented here are the first to demonstrate that in contrast to covalent DNA adducts formed by ellipticine, the adducts generated by formaldehyde-mediated covalent binding of doxorubicin to DNA are not detectable by the ³²P-postlabeling assay. No DNA adducts were detectable in either *in vitro*, in incubations of DNA with doxorubicin or in DNA of neuroblastoma cells treated with this drug. The results of this and our previous study⁷ also suggest that covalent binding of ellipticine in DNA of UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines is the predominant mechanism responsible for cytotoxicity of this drug to these cells. The mechanism of toxicity of doxorubicin to neuroblastoma cells has, however, not been resolved by this study and the development of novel methods identifying covalent doxorubicin-derived DNA adducts is the major challenge for further research.

ABBREVIATIONS

CYP, cytochrome P450; DMSO, dimethyl sulfoxide; IMDM, Iscove's modified Dulbecco's medium; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; PEI-cellulose, polyethylenimine-cellulose; RAL, relative adduct labeling; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography.

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