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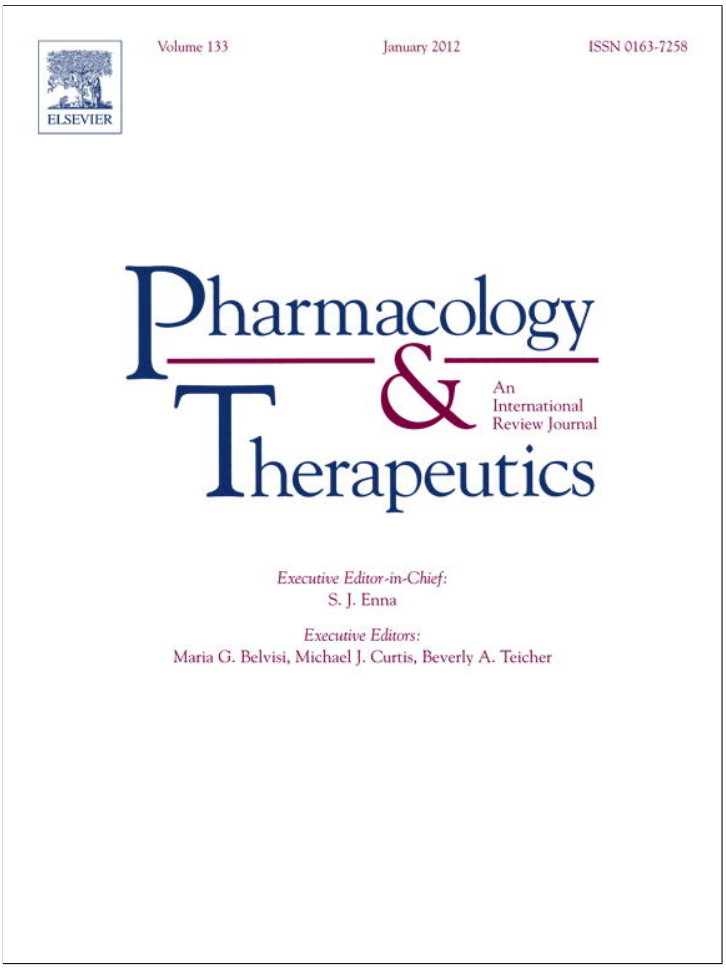

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Anthracyclines and ellipticines as DNA-damaging anticancer drugs: Recent advances

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ABSTRACT

Over the past forty years, anthracyclines and ellipticines have attracted attention as promising cytostatics. In this review, we focus on their mechanisms of cytoxicity, DNA-damaging effects and adverse side-effects. We also summarize ways to enhance the therapeutic effects of these drugs together with a decrease in their adverse effects. Current drug design strategies are focused on drug bioavailability and their tissue targeting, whereas drug delivery to specific intracellular compartments is rarely addressed. Therefore, therapies utilizing the antineoplastic activities of anthracyclines and ellipticines combined with novel strategies such as nanotechnologies for safer drug delivery, as well as strategies based on gene therapy, could significantly contribute to medical practice.

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1. DNA-adducts and their biological significance

The potential of chemicals (drugs) to induce toxic effects and DNA damage depends on their metabolism. If the administered drug has a direct toxicity of its own (or acts directly on DNA or a receptor to produce toxicity), then metabolism of the drug, mediated by many enzymes such as cytochromes P450 (CYP), peroxidases or NAD(P)H:quinoneoxidoreductase, may reduce its toxicity if the product generated by such enzymes has less inherent toxicity. Another case is the transformation of an administered drug to another that either (i) binds covalently to macromolecules such as DNA (usually because of its electrophilic or other reactive nature) or (ii) otherwise interacts with a target to cause toxicity. Examples (i) and (ii) are (usually) distinguished by (i) their capability for a genotoxic response vs. (ii) a tendency to act by causing increased cell proliferation, although these two phenomena are not mutually exclusive. The list of potential DNA, lipid, and protein targets with reactive electrophiles and radicals is extensive. With DNA, understanding at least some of the most relevant gene responses and their mechanisms is becoming possible.

Genetic damage that produces a heritable loss of growth control, differentiation or apoptosis comprises a major mechanism of carcinogenesis. Exposure to drugs having genotoxic side effects results in structural damage to DNA, which occurs primarily as the covalent binding of chemicals and is referred to as drug–DNA adduct formation (Hemminki et al., 1994). Numerous types of DNA adducts have been identified. Generally, nitrogen and oxygen atoms on the bases

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are active sites. There have been reactions described on the N^2, N-3, O^6 and N-1 sites of guanine, N-1, N-3, N^6 and N-7 sites of adenine, O^2, N-3 and N^4 sites of cytosine and O^2 and O^4 sites of thymine. There are also some reactions known on the phosphate and deoxyribose moieties. Guanine is the most reactive base of the four DNA bases. Guanines modified on N^2, O^6 and C-8 hydroxylated guanine [8-hydroxyguanine (8-OH-Gua)] are well-known adducts (Fraga et al., 1990; Shigenaga & Ames, 1991). Exposure to some aldehydes and ultraviolet rays is responsible for the formation of cross-links between the amino groups of bases in DNA. In addition, intrastrand cross-linking can cause miscopy of DNA (Matsuda et al., 1998). The above-mentioned DNA damage is generally considered to be causative and directly related to tumor formation (Lutz, 1986; Poirier, 1996; Otteneder & Lutz, 1999; Hemminki et al., 2000; Poirier et al., 2000; Wiencke, 2002). Indeed, associations have been observed between DNA adduct formation, mutagenesis (Poirier et al., 2000; Wiencke, 2002), and tumorigenesis (Otteneder & Lutz, 1999; Poirier et al., 2000), while reductions in DNA adduct levels have been associated with chemoprevention (Wiencke, 2002). Therefore, it is not surprising that investigation of the mechanisms responsible for the toxicity of DNA adducts, which are essential for cancer development, attracts the attention of many laboratories.

DNA damage is, however, also employed for cancer treatment. In other words, many efficient anticancer drugs are based on the modification of DNA in cancer cells. Indeed, several cytostatics are capable of forming DNA-adducts. The aim of this review is to furnish information about two similar classes of cytostatics acting as DNA-damaging agents, doxorubicin and its derivatives and ellipticines. In addition, their metabolism and mechanisms of action based on interactions with DNA and other mechanisms (interaction with topoisomerases II, generation of free radicals, etc.) are reviewed, along with data showing their biological connection to low molecular mass protein metallothionein.

2. Ellipticines

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole, NSC 69178, Fig. 1), an alkaloid isolated from *Apocynaceae* plants (i.e., *Ochrosia borbonica*, *Excavatia coccinea*), is one of the simplest naturally occurring alkaloids and has a planar structure (Goodwin et al., 1959). It was first isolated in 1959 from leaves of the evergreen tree *Ochrosia elliptica*, which grows wild in Oceania (Goodwin et al., 1959). In preclinical experiments and clinical trials, this compound and several of its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N^2-methylellipticinium, 9-chloro-N^2-methylellipticinium, 9-methoxy-N^2-methylellipticinium and 9-dimethyl-amino-ethoxy-ellipticine) exhibited significant antitumor and anti-HIV activities (Dalton et al., 1967; Rouesse et al., 1985; Mathe et al., 1998). They showed activity against cancer cell lines such as leukemias, melanoma, sarcomas, myelomas, lymphomas, colon adenocarcinoma, lung carcinoma, diverse brain tumors, osteosarcoma, breast cancer and neuroblastomas, which were killed at concentrations ranging from 10^{-10} to 10^{-6} M (for an overview, see Arguello et al., 1998; Garbett & Graves, 2004; Stiborova et al., 2006b; Tian et al., 2008). One of the ellipticine derivatives, 9-hydroxy-N^2-methylellipticine (NSC 264-137), selected for a phase I and later a phase II trial in human cancer, seems to particularly improve the condition of patients suffering from breast cancer (Dodion et al., 1982), especially after it has metastasized to the bone (Paoletti et al., 1980; Juret et al., 1982; Rouesse et al., 1985). Activity against anaplastic thyroid carcinoma and ovarian carcinoma has also been observed.

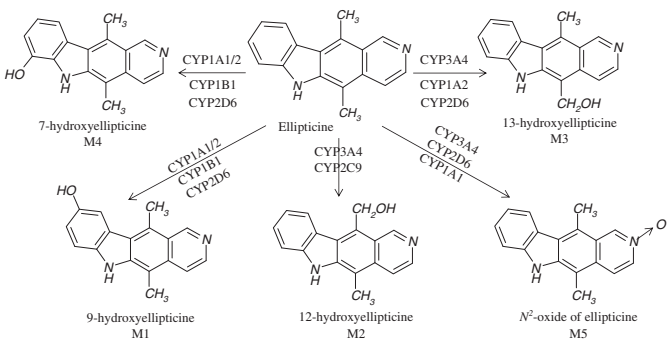

Fig. 1. Metabolism of ellipticine by human CYPs showing the metabolites and CYPs responsible for their formation.

The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited side effects and, in particular, their lack of hematologic and hepatic toxicity (Auclair, 1987). In patients that received a weekly 80 mg/m^2 of 9-hydroxy-N^2-methylellipticine, no renal trouble was observed during the first year, but 2 deaths from renal insufficiency occurred during the 15th and 18th months of treatment. Nevertheless, the most frequent side effect was digestive troubles (nausea and vomiting in one-third of the patients), which rarely results in termination of the treatment, hypertension, muscular cramps, chronic fatigue (which can be very pronounced), mouth dryness, mycosis of the tongue and esophagus (Paoletti et al., 1980). In addition, the mutagenicity of ellipticines should be evaluated as a potential risk factor. Namely, most ellipticines are mutagenic to *Salmonella typhimurium* Ames tester strains, bacteriophage T4, *Neurospora crassa*, and mammalian cells, as well as induce prophage lambda in *Escherichia coli* (for an overview, see Stiborova et al., 2001). In addition, ellipticine provided positive results in a mouse lymphoma assay (Moore et al., 1987), in the forward gene mutation at the hypoxanthine-guanine phosphoribosyltransferase locus in Chinese hamster ovary cells (DeMarini et al., 1983), and in the induction of sister-chromatid exchanges in cultured mammalian cells (Noviello et al., 1994).

2.1. Mechanisms of the cytotoxicity and anticancer activity of ellipticines

The anticancer drug ellipticine and its derivatives act via combined mechanisms of cell cycle arrest and induction of the apoptotic pathway. Ellipticine has been reported (i) to arrest cell cycle progression by regulating the expression of cyclin B1 and Cdc2 as well as phosphorylation of Cdc2 in human breast cancer cell lines (Kuo et al., 2005a, 2005b), (ii) to induce apoptotic cell death through the generation of cytotoxic free radicals, activation of the Fas/Fas ligand system and regulation of Bcl-2 family proteins in human breast cancer and hepatocellular carcinoma cells (Kuo et al., 2005a, 2005b, 2006), (iii) to induce an increase in wild-type p53, rescue mutant p53 activity and (iv) to cause initiation of the mitochondrial apoptosis pathway (Kuo et al., 2005a, 2005b, 2006). Numerous studies have reported the involvement of p53 tumor suppressor protein in the cytotoxic effects of ellipticine (Sugikawa et al., 1999; Peng et al., 2003; Kuo et al., 2005a). 9-Hydroxyellipticine treatment caused an induction of apoptosis in the G1 phase of the cell cycle in mutant p53-transfected Saos-2 cells, but not in p53-deficient parental Saos-2 cells (Sugikawa et al., 1999). Ellipticine and 9-hydroxyellipticine caused selective inhibition of p53 protein phosphorylation via kinase inhibition in several human cancer cell lines such as Lewis lung carcinoma and human colon cancer cell line SW480 (Ohashi et al., 1995), and this correlated with their cytotoxic activity. Moreover, the accumulation of dephosphorylated mutant p53 might induce apoptosis (Ohashi et al., 1995). Ellipticine has also been found to restore the transcription function of mutant p53. This property may contribute to the selectivity of ellipticine-derived compounds against tumor cell lines expressing mutant p53 (Peng et al., 2003). In human breast adenocarcinoma MCF-7 cells, ellipticine causes G2/M phase arrest associated with an increase in the protein expression of p53 and KIP1/p27, but not WAF1/p21, and growth inhibition by this

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compound is also induced by mitochondrial proapoptotic pathways in these cells (Kuo et al., 2005b). Treatment of human neuroblastoma cell lines with ellipticine resulted in growth inhibition associated with apoptosis induction (Poljakova et al., 2009). Another important result of the study with neuroblastoma cells was the finding that these cancer cells can become resistant to ellipticine only after prolonged treatment and to a much lower extent than to doxorubicin, cisplatin or vincristine (Poljakova et al., 2009). Because drug resistance is a general feature of high risk neuroblastoma and arises in the majority of patients, this finding is a promising result that might lead to the development of new neuroblastoma therapies by substituting or combining current anticancer drugs with ellipticine (Poljakova et al., 2009). Preliminary experiments suggest that ellipticine resistance is not dependent on P-glycoprotein expression, which is the case in the UKF-NB-4 cell line resistant to doxorubicin and vincristine (Bedrnicek et al., 2005). The IMR-32 and UKF-NB-4 cell lines accumulated in the S phase of the cell cycle, whereas different effects of ellipticine on the cell cycle of UKF-NB-3 cells were detected; an arrest in the G0/G1 and G2/M phases was found in this neuroblastoma line (Poljakova et al., 2009).

However, the precise molecular mechanism responsible for these effects has not yet been explained. Chemotherapy-induced cell cycle arrest was shown to result from DNA damage caused by a variety of chemotherapeutics. In the case of ellipticine, it has been suggested that the prevalent DNA-mediated mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA (Singh et al., 1994) and (ii) interaction with DNA topoisomerase II activity (Auclair, 1987; Fosse et al., 1992; Froelich-Ammon et al., 1995). The size and shape of the ellipticine chromophore closely resemble those of a purine–pyrimidine complementary base pair, providing favorable conditions for its intercalation in double-stranded DNA. Furthermore, the polycyclic aromatic character of the molecule may result in tight interactions with appropriately conformed hydrophobic regions in DNA. Interactions between the methyl groups of the drug and the thymine bases at the intercalation site appear to be important in determining the orientational preferences of the drug (Singh et al., 1994).

The mechanism of ellipticine action as a possible inhibitor of topoisomerase II has also been extensively studied (Froelich-Ammon et al., 1995). Ellipticine acts by stimulating topoisomerase II-mediated DNA breakage. It is likely that formation of a ternary complex between topoisomerase II, DNA, and drug is critical for nucleic acid breakage and subsequent cell death. Topoisomerase II has been identified as one of the primary cellular targets of the drug. Furthermore, ellipticine did not inhibit enzyme-mediated DNA religation, suggesting that it stimulates DNA breakage by enhancing the forward rate of cleavage. Froelich-Ammon and co-workers postulated that ellipticine enters the ternary complex through its prior association with either DNA or the enzyme and does not require the presence of a preformed topoisomerase II–DNA complex (Froelich-Ammon et al., 1995).

In addition, ellipticine also uncouples mitochondrial oxidative phosphorylation (Schwaller et al., 1995) and thereby disrupts the energy balance of cells. In the case of ellipticine derivative 9-dimethyl-amino-ethoxy-ellipticine (NSC 338258, EPED3) in myeloma cells, NSC 338258-induced cell cycle arrest and an apoptotic progression that appeared to be a consequence of the instantaneous effect of the drug on cytoplasmic organelles, particularly mitochondria. Disruption of mitochondria and cytoplasmic distribution of cytochrome *c* initiated the intracellular proteolytic cascade via the intrinsic apoptotic pathway. Moreover, NSC 338258 was able to induce apoptosis in myeloma cells with de novo or acquired resistance to commonly administered antimyeloma agents (Tian et al., 2008). Studies on the mechanisms of the cytotoxicity and anticancer activity of ellipticines have also shown that these activities might be due to an induction of endoplasmic reticulum stress (Hagg et al., 2004).

2.2. Covalent binding of ellipticine to DNA: a novel mode of ellipticine action

It is evident that the explanations of anticancer activity mentioned above are based on mechanisms of nonspecific drug actions. Intercalation of ellipticine into DNA and inhibition of topoisomerases II occur in all cell types irrespective of their metabolic capacity, because of the general chemical properties of this drug and its affinity for DNA (Garbett & Graves, 2004; Stiborova et al., 2006b). In addition, the other ellipticine effects described above, and the transport of highly hydrophobic ellipticine molecules across cell membranes into cells (including both tumor and healthy cells), are nonspecific. However, this sharply contrasts with the specificity of antineoplastic activity of ellipticines against only cancer diseases. The specificity of antitumor activity of ellipticines should be a consequence of other mechanisms of action, which have not yet been fully elucidated. 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 (Fig. 1) (Stiborova et al., 2001, 2003a, 2003b, 2004, 2006a, 2006b, 2007a, 2008; Kotrbova et al., 2006; Poljakova et al., 2006, 2007, 2009), suggesting a third mechanism of action.

Using a ^{32}P-postlabeling method, we have found that during ellipticine oxidation by CYPs and peroxidases, two major and several minor ellipticine-derived adducts are generated in DNA (Fig. 2) (Stiborova et al., 2003b, 2007a, 2007b, 2008; Kotrbova et al., 2006; Poljakova et al., 2006). Human and rat CYP1A, 1B1 and 3A, which are expressed at higher levels in tumors sensitive to ellipticine (i.e., breast cancer) than in peritumoral tissues (El-Rayes et al., 2003; Patterson et al., 1999), are the predominant enzymes catalyzing the oxidation of ellipticine in vitro to metabolites that are either excreted (7-hydroxy- and 9-hydroxyellipticine) or that form DNA adducts (12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide) (see Figs. 1 and 2A, F, G and H showing the adducts formed by ellipticine activated with CYP3A4 by 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide, respectively) (Stiborova et al., 2004, 2006b, 2007a, 2008). Of the mammalian peroxidases, bovine lactoperoxidase (LPO), human myeloperoxidase (MPO), which is highly expressed in acute myeloid leukemia blasts, bovine COX-1, and human cyclooxygenase (COX)-2 efficiently generate ellipticine-derived DNA adducts (see Figs. 2B, C, D and E for adducts formed by ellipticine activated with LPO, MPO, COX-1 and COX-2, respectively) (Poljakova et al., 2006; Stiborova et al., 2007a). CYP- and/or peroxidase-mediated ellipticine-DNA adducts have also been detected in rats and mice in vivo (Figs. 2I and J) (Stiborova et al., 2007b; Stiborova et al., 2008). Deoxyguanosine was identified as the target base to which ellipticine metabolites generated by CYPs and/or peroxidases (12-hydroxy- and 13-hydroxyellipticine) are bound (Stiborova et al., 2004), forming the two major ellipticine-derived DNA adducts (adduct spots 1 and 2 in Figs. 2 and 3) in vitro (Stiborova et al., 2004, 2007a). These adducts are formed from two reactive species, ellipticine-13-ylium and ellipticine-12-ylium (Fig. 3), which we had suggested earlier to react with one of the nucleophilic centers in the deoxyguanosine residue of DNA (e.g., the exocyclic amino group of guanine, Fig. 3) (Poljakova et al., 2006; Stiborova et al., 2007a; Moserova et al., 2008). The low amount of each DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine, 12-hydroxyellipticine or ellipticine N^2-oxide (Figs. 2 F–H), however, prevented their further structural characterization. Synthetic approaches are currently being employed in our laboratory to prepare authentic ellipticine-DNA adduct standards (Dracinsky et al., 2007; Moserova et al., 2008). The same DNA adducts were also detected in human hepatic and renal microsomes (Stiborova et al., 2007a), as well as in cells in culture expressing enzymes activating ellipticine (CYP1A1, CYP3A4, COX-1 and MPO), such as human breast adenocarcinoma MCF-7 cells (Fig. 2L) (Borek-Dohalska et al., 2004), leukemia HL-60 and CCRF-CEM cells (Figs. 2M

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and N) (Poljakova et al., 2007), IMR-32, UKF-NB-3 and UKF-NB-4 (Fig. 2O) neuroblastoma cell lines (Poljakova et al., 2009), a glioblastoma U87MG cell line (Fig. 2P) (Stiborova et al., 2007a), BHT-101, B-CPAP and 8505-C thyroid gland cancer cell lines and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (Frei et al., 2002). 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.

It is not possible to demonstrate if the antitumor, cytostatic and/or genotoxic activities of ellipticine are related to only one or several of the DNA damaging effects. Recently, we demonstrated that the cytotoxicity elicited by ellipticine in Chinese hamster fibroblast V79 cells does not seem to be dependent on CYP expression (Frei et al., 2002). Acute toxicity in these cells could be caused by the parent compound itself, perhaps by uncoupling mitochondrial oxidative phosphorylation, thereby disrupting the cellular energy balance (Schwaller et al., 1995), or by CYP-independent metabolites. With respect to the cytotoxic activity of ellipticine against cancer cells, however, other properties such as mutagenicity caused by DNA adducts might be relevant. Indeed, in promyelocytic HL-60 cells and T-cell leukemia CCRF-CEM cells, the cytotoxicity of ellipticine correlates with the levels of ellipticine-derived DNA adducts generated in these cells (Poljakova et al., 2007). The different levels of enzymes activating ellipticine to species that form DNA adducts in leukemia cells used in this study might explain this finding. While the CYP1A1 and COX-1 enzymes are expressed in both HL-60 cells and CCRF-CEM cells, HL-60 cells also express high levels of another activation enzyme, MPO. This feature could explain the higher DNA adduct levels and cytotoxicity of ellipticine in this promyelotic leukemia line than in CCRF-CEM lymphoblastic leukemia cells (Poljakova et al., 2007).

Likewise, Rekha and Sladek (1997) demonstrated that the antineoplastic activity of ellipticine in MCF-7 cells depends on the levels of CYP enzymes converting ellipticine to DNA-binding species. These authors showed that MCF-7 cells treated with 3-methylcholanthrene transiently expressed elevated levels of CYP1A and were transiently much more sensitive to ellipticine. CYP1A also converts ellipticine to species that bind to DNA (Frei et al., 2002), and in an earlier study, we found typical ellipticine-DNA adducts in these cells (Borek-Dohalska et al., 2004). The CYP-dependent higher sensitivity of MCF-7 cells to ellipticine observed by these authors might, therefore, be explained by ellipticine-DNA adduct formation. This explanation was also confirmed by the finding that ellipticine itself is able to induce several CYPs, the enzymes that activate ellipticine to form DNA adducts. Induction of CYP1A1/2 enzymes has been found in rats (Aimova et al., 2007), whereas CYP1A1, 1B1 and 3A4 have been found in human U87MG glioblastoma cells treated with this drug (Martinkova et al., 2009). By inducing these CYP enzymes, ellipticine increases its own metabolism, leading to both an activation of this drug to reactive species that form DNA adducts (Aimova et al., 2007) and to a detoxification of metabolites (Aimova et al., 2007), thereby modulating its pharmacological and/or genotoxic potential to some extent.

Another important feature relating the expression of CYP and the antineoplastic activity of ellipticine was detected in MCF-7 cells selected for resistance to adriamycin (Adr^R MCF-7) and exhibiting the phenotype of multidrug resistance (MDR) (Ivy et al., 1988). Ivy et al. (1988) postulated that the MDR property of Adr^R MCF-7 cells involves several biochemical and genetic changes (Ivy et al., 1988). One of these changes is a regulatory defect at the level of CYP1A1 mRNA, resulting in lower CYP1A1-mediated metabolism of xenobiotics in these cells. Adr^R MCF-7 cells are cross-resistant to ellipticine (Ivy et al., 1988), which might be explained by a decrease in the CYP1A1-dependent activation of ellipticine.

In neuroblastoma cells, a correlation was determined between the effect of ellipticine (expressed as IC_{50} values) and the formation of ellipticine-DNA adducts in IMR-32 and UKF-NB-4 cells (Poljakova et al., 2009). These findings suggest that the cytotoxic activity of ellipticine to these two neuroblastoma cell lines is also a consequence of the formation of ellipticine-DNA adducts. The role of ellipticine-DNA adduct formation in cytotoxicity was further supported by the finding that a decrease in the levels of these adducts in IMR-32 and UKF-NB-4 cells under hypoxic conditions, which inhibits CYP-mediated ellipticine activation, corresponded to a decrease in toxicity of ellipticine under these conditions.

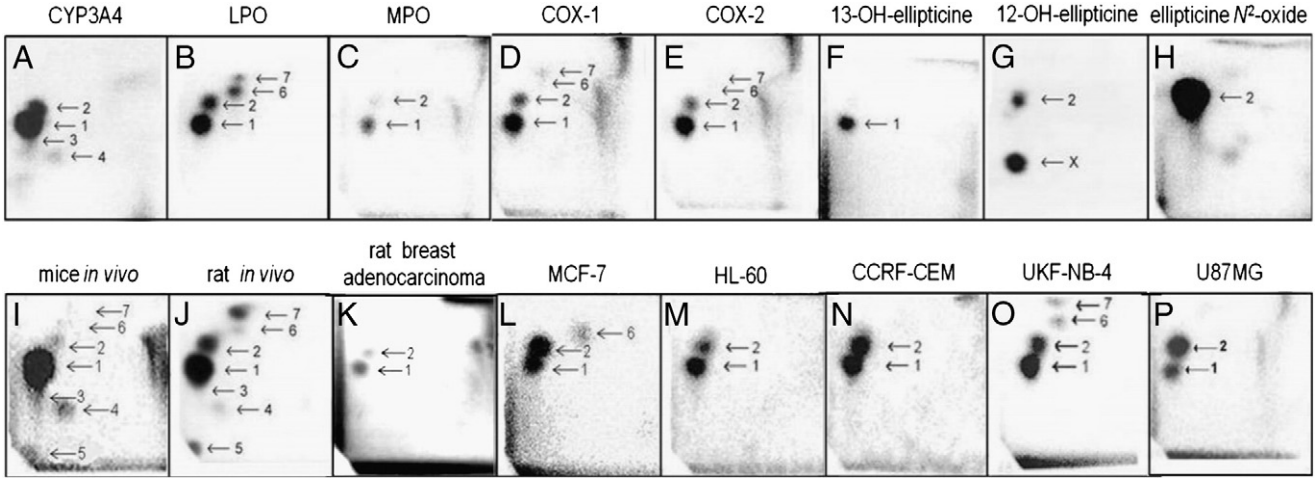

Fig. 2. Autoradiographic profiles of ellipticine-derived DNA adducts analyzed with a ^{32}P-postlabeling assay. Adduct profiles obtained from calf thymus DNA reacted with ellipticine (100 μM) and CYP3A4 (**A**), bovine LPO (**B**), human MPO (**C**), bovine COX-1 (**D**), and human COX-2 (**E**), from calf thymus DNA reacted with 13-hydroxyellipticine (**F**), 12-hydroxyellipticine (**G**), and ellipticine N^2-oxide (**H**), from liver DNA of C57BL/6 mice treated i.p. with 10 mg ellipticine per kilogram body weight (**I**), from liver DNA of Wistar rats treated i.p. with 40 mg ellipticine per kilogram body weight (**J**), from DNA of breast adenocarcinoma of Wistar rats treated i.p. with 4 mg ellipticine per kilogram body weight (**K**), from DNA of breast adenocarcinoma MCF-7 cells (**L**), leukemia HL-60 (**M**) and CCRF-CEM cells (**N**), neuroblastoma UK-NB-4 cells (**O**) and glioblastoma U87MG cells (**P**) treated with 10 μM ellipticine. Adduct spots 1–7 correspond to the ellipticine-derived DNA adducts. Aside from adduct 2 formed by 12-hydroxyellipticine, another strong adduct (spot X in panel G — calf thymus DNA reacted with 12-hydroxyellipticine) that was not found in any other activation systems or in vivo was generated.
Adapted and modified according to Stiborova et al., 2011.

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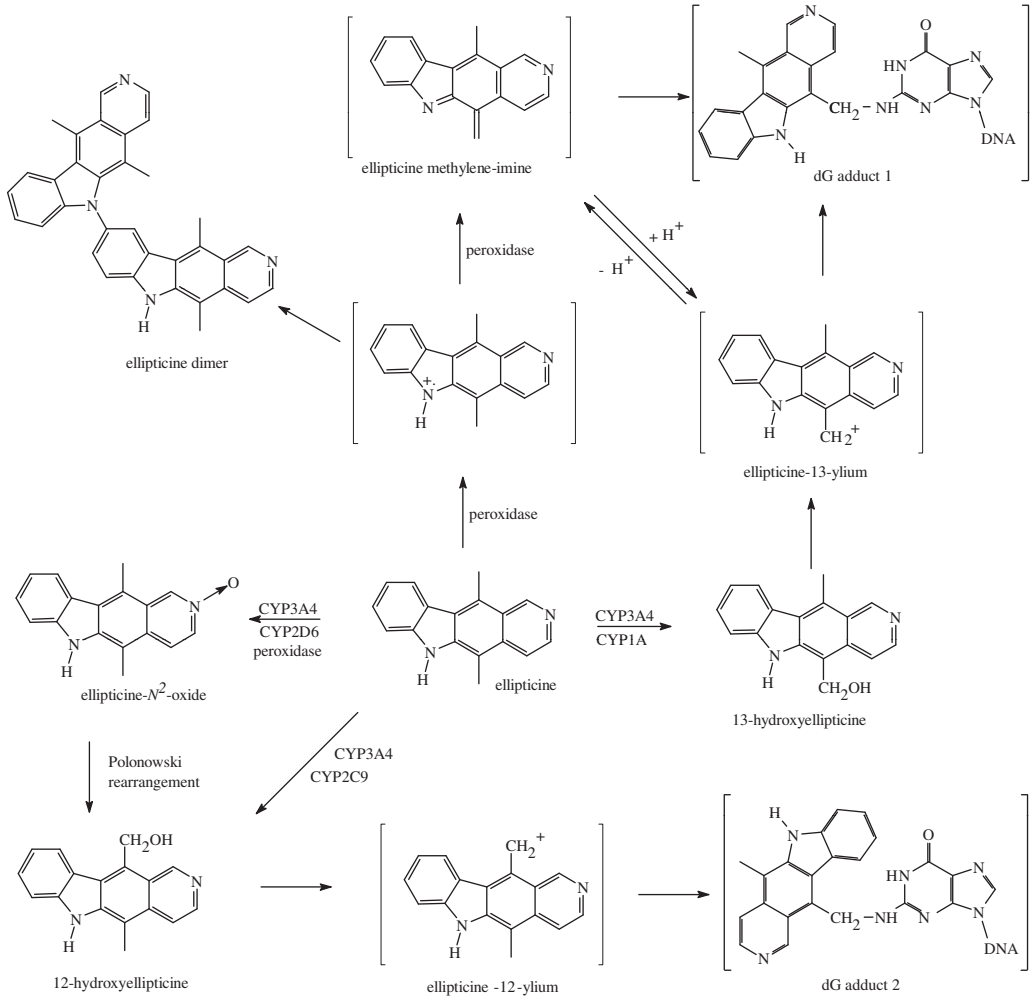

Fig. 3. Metabolism of ellipticine by peroxidases and human CYPs showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as the ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

A number of DNA-damaging agents have been shown to inhibit cell growth by arrest in the G1 to S checkpoint (Khan et al., 1999; Simoes et al., 2008). This cell cycle arrest is thought to be an important cellular defense mechanism that prevents replication of damaged DNA. We found that exposure to ellipticine caused an accumulation of IMR-32 and UKF-NB-4 cells in S phase. It is tempting to speculate that the mechanism of the S phase delay is the inability of the DNA polymerase complex to replicate over ellipticine-induced DNA adducts. Namely, it has been shown that DNA damage blocks DNA replication and/or transcription by polymerase (Roos & Kaina, 2006; Simoes et al., 2008). In addition, inhibition of DNA replication has also been implicated as a proximate initiator of apoptosis (Roos & Kaina, 2006), which we found in neuroblastoma cells (Poljakova et al., 2009).

Taken together, the activities and expression levels of CYP enzymes, which effectively activate ellipticine to metabolites that form DNA adducts, may be important factors in the specificity of ellipticine for acute myeloid leukemia, breast cancer, glioblastoma and neuroblastoma. Moreover, we recently found that ellipticine-DNA adducts are also formed in cancer tissue in vivo (Stiborova et al., 2011). Ellipticine-derived DNA adducts are detectable not only in healthy organs of rats and mice exposed to ellipticine (Figs. 2I and J) (Stiborova et al., 2007b, 2008), but also in the target tissue, mammary tumors (Fig. 2K) (Stiborova et al., 2011). Furthermore, to better understand the role of ellipticine-DNA adducts in the pharmacological efficacy of cancer treatment and the genotoxic side effects of the drug, we analyzed the dose dependence and persistence of ellipticine-DNA adducts in non-target tissues (liver, lung, kidney, spleen, heart and brain) of rats treated with ellipticine, because the animal model can mimic the bioactivation of ellipticine in humans. Only very low levels of adducts are retained in the DNA of these non-target tissues. In addition, not all of the ellipticine-DNA adducts persisted in the tissues analyzed in the study (only adducts 1, 2, 4 and 5) (Stiborova et al., 2007b). This finding demonstrates that healthy tissues of rats treated with ellipticine possess effective repair systems to remove certain lesions and suggests a relatively low impact of the genotoxic side effects of ellipticine during cancer treatment in humans.

3. Anthracyclines

3.1. Chemical properties of anthracyclines

Daunorubicin, (8S-cis)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-alpha-L-lyxo-hexopyranosyl) oxy]7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione, the first used anthracycline antibiotic, was isolated from cultures of *Streptomyces peucetius* in Italy in 1963 (Grein et al., 1963). In preclinical experiments and clinical trials, it showed activity against acute leukemias. *Doxorubicin*, isolated shortly afterwards, showed demonstrable activity against a wider range of tumors in children, including soft tissue and bone sarcomas, nephroblastoma and lymphomas, as well as lymphoblastic and myeloid leukemia (Bonadonna et al., 1969). Anthracyclines are commonly used in the treatment of a number of diverse malignant

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tumors, including acute lymphoblastic and myeloid leukemia, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, lung, ovarian, gastric, thyroid and breast carcinoma, soft tissue sarcomas, osteosarcoma, neuroblastoma, and nephroblastoma, among others (Takimoto, 2005). There have been several newer anthracyclines developed, such as *epirubicin*, a less cardiotoxic doxorubicin analogue with activity in sarcomas, breast and gastric cancer; *idarubicin*, an analogue with increased efficacy in acute myeloid leukemia; and *valrubicin*, an anthracycline used for the intravesical treatment of bladder cancer.

A major problem with the clinical use of anthracyclines, in addition to adverse side effects common to all cytostatics (like myelosupression, nausea and vomiting, mouth ulcers, local agressivity and alopecia), is their cardiotoxicity. Cardiotoxicity limits the administration of doxorubicin exceeding an accumulated dose of ~450–550 mg/m^2 (Singal et al., 1997). The toxic effects of anthracyclines to cardiomyocytes are not the result of inhibition of DNA synthesis, because these cells do not replicate (Myers, 1998). Although the mechanisms of anthracycline cardiotoxicity are not fully understood, a number of observations suggest that interactions of anthracyclines with iron ions are of great importance. The redox state of iron ions can be converted between the iron(II) and iron(III) by interaction with anthracyclines, generating toxic reactive oxygen species (ROS), which cause DNA damage and induce apoptosis. Cardiac tissue is vulnerable to free radical damage because of the low activity of antioxidant enzyme systems in cardiomyocytes (Xu et al., 2005). The most successful strategy to decrease the cardiotoxicity of anthracyclines involves the liposomal encapsulation of drugs, which changes their tissue distribution and pharmacokinetics. The cardiac safety of liposomal daunorubicin, liposomal doxorubicin, and pegylated liposomal doxorubicin has been studied in a number of clinical trials, which indicated that the risk of anthracycline-induced cardiotoxicity is considerably lower with liposomal anthracyclines (Safra, 2003).

All anthracyclines share a quinone containing a rigid planar aromatic ring structure bound by a glycosidic bond to the amino sugar daunosamine (Fig. 4A). Doxorubicin and daunorubicin have the same aglyconic and sugar moieties. The aglycone consists of a tetracyclic ring with adjacent quinone–hydroquinone groups in rings B and C, a methoxy substituent at C-4 in ring D, and a short side chain at C-9 with a carbonyl at C-13. The sugar daunosamine is attached by a glycosidic bond to the C-7 position of ring A and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl moiety. The only difference between doxorubicin and daunorubicin is that the side chain of doxorubicin terminates with a primary alcohol, whereas that of daunorubicin terminates with a methyl. This minor difference, however, has important consequences on the spectrum of activity and pharmacokinetics of the compounds (Minotti et al., 2004). Epirubicin is obtained by an axial-to-equatorial epimerization of the hydroxyl group at C-4′ in daunosamine. This positional change has little effect on the mode of action. It introduces pharmacokinetic and metabolic changes like increased volume of distribution, 4-*O*-glucuronidation, and consequently, an enhanced total body clearance or shorter terminal half-life (Robert & Gianni, 1993; Danesi et al., 2002). Because of these kinetic and metabolic changes, epirubicin has been used at cumulative doses, almost 2-fold higher than doses of doxorubicin, resulting in equal activity, but not increased cardiotoxicity (Robert & Gianni, 1993). Idarubicin is an analogue obtained from daunorubicin after removal of the 4-methoxy group in ring D. This drug is active in acute myelogenous leukemia, multiple myeloma, non-Hodgkin's lymphoma, and breast cancer (Borchmann et al., 1997). The broader spectrum of activity of idarubicin compared with daunorubicin may be attributed to its increased lipophilicity and cellular uptake, and improved stabilization of its ternary drug–topoisomerase II–DNA complex, one of major mechanisms of anthracycline activity (see later) (Binaschi et al., 2001). In addition, idarubicin may be administered orally with ~10 to 30% bioavailability (Toffoli et al., 2000). In vitro studies have indicated that idarubicin might be more effective than daunorubicin in tumor cell lines displaying the multidrug resistance phenotype caused by overexpression of the MDR-1 gene (Toffoli et al., 1994; Jonsson-Videsater et al., 2003).

3.2. Biological effects of anthracyclines

Only a few additional anthracyclines have obtained clinical approval; these include pirarubicin, aclacinomycin A (aclarubicin), and mitoxantrone (a substituted aglyconic anthraquinone) (for structures, see Fig. 4B). Both pirarubicin and aclarubicin demonstrate only modest improvements over doxorubicin and daunorubicin in terms of multidrug resistance (Lothstein et al., 2001). Pirarubicin, a 4-tetrahydropyranyl doxorubicin, has been reported to induce much less cardiotoxicity than doxorubicin in animal models (Koh et al., 2002), but clinical studies have indicated that it may cause severe cardiac dysfunction in humans (Dhingra et al., 1995; Niitsu et al., 1998). Aclarubicin, a trisaccharide anthracycline, was shown to be active (with tolerable side effects) in adult patients with acute myeloblastic leukemia (Case et al., 1987; Wojnar et al., 1989). However, aclarubicin induced late cardiac events (Dabich et al., 1986) and proved to be inactive in women with metastatic breast cancer (Natale et al., 1993). Mitoxantrone is active in breast cancer, non-Hodgkin's lymphoma, acute promyelocytic and myelogenous leukemias, as well as androgen-independent prostate cancer. Early reports indicated that mitoxantrone was less cardiotoxic than other anthracyclines (Estorch et al., 1993), but this conclusion has been corrected in more recent studies (Thomas et al., 2002).

As a consequence of the diverse molecular effects of anthracyclines, the mechanism of their cytotoxicity may involve multiple pathways, but the precise mechanism of their action remains obscure because of the complexity of these mechanisms. The following mechanisms have been considered: 1) intercalation into DNA, leading to inhibited synthesis of macromolecules (DNA, RNA and proteins); 2) generation of free radicals, leading to DNA damage and/or lipid peroxidation; 3) DNA binding and alkylation; 4) DNA cross-linking; 5) interference with DNA unwinding or DNA strand separation; 6) inhibition of helicase activity; 7) direct membrane effects; and 8) inhibition of topoisomerase IIα (Minotti et al., 2004). The antiangiogenic effects of the anthracyclines may explain, at least in part, their antitumor properties. Daunorubicin, doxorubicin, and epirubicin inhibited capillary-like tube formation in an in vitro system of angiogenesis using human umbilical vein endothelial cells, decreased vascular density and inhibited collagenous protein biosynthesis in a chicken chorioallantoic membrane model of angiogenesis (Maragoudakis et al., 1994).

There is compelling evidence that cellular DNA is the primary target of anthracyclines. The anthraquinone ring intercalates between DNA base pairs, with its long axis nearly perpendicular to the axis of the double helix. One of the rings acts as an anchor and stabilizes the complex through hydrogen bond interactions as the daunosamine sugar lies in the minor groove. The occurrence of a single positive charge on daunomycin contributes electrostatically to the binding (Rabbani et al., 2005). The concentrations of anthracyclines used in clinical practice caused formation of protein-associated DNA single- and double-strand breaks that were affected by topoisomerase II inhibition. DNA lesions caused by the formation of free radicals and reactivity on the DNA backbone occurred when cells were treated with doxorubicin at concentrations that were too high for patient use (Gewirtz, 1999). However, at clinically relevant concentrations, anthracyclines do not induce lipid peroxidation in cancer cells (Kiyomiya et al., 2001).

Doxorubicin forms unstable covalent adducts with DNA when it is activated with NAD(P)H-dependent oxidoreductases and transition metals. Two types of covalent DNA modification by doxorubicin have been described: (i) cross-links, which seem to be more stable, and (ii) less stable drug–DNA adducts. It was found that in cells, iron-mediated free radical reactions enable doxorubicin to produce

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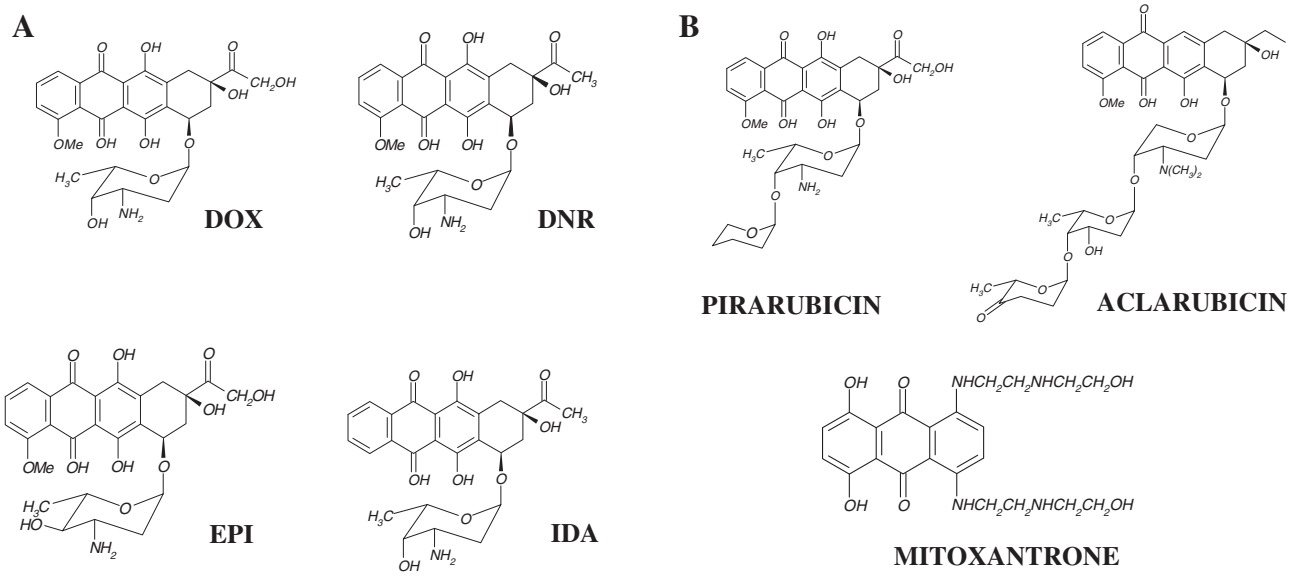

Fig. 4. (**A**) Structures of doxorubicin, daunorubicin, epirubicin and idarubicin. Residues highlighted with gray indicate that the side chain of daunorubicin or idarubicin terminates with a methyl in place of a primary alcohol compared with doxorubicin or epirubicin. Dotted arrows indicate structural modifications in epirubicin compared with doxorubicin (axial to equatorial epimerization of the hydroxyl group at C-4′ in daunosamine), or in idarubicin compared with daunorubicin (lack of a methoxy group at C-4 in ring D) (Minotti et al., 2004). (**B**) Structures of pirarubicin, aclarubicin, and mitoxantrone.

formaldehyde from sources of carbon like spermine and lipids (Taatjes & Koch, 2001). High levels of formaldehyde have been detected in cancer cells sensitive to anthracyclines, but not in resistant ones (Kato et al., 2001). Doxorubicin and endogenous formaldehyde form a conjugate of two anthracycline molecules with three methylene groups, two forming oxazolidine rings and one binding the oxazolidines together at their 3′-amino nitrogens (Fig. 5). This conjugate may hydrolyze to produce an active monomeric metabolite in which the carbon of formaldehyde is recovered in the form of a Schiff's base at the amino group of daunosamine. These reactions occur only with anthracyclines containing the 3′-amino group (epirubicin and daunorubicin) (Cutts et al., 2003). Anthracycline-formaldehyde conjugates intercalate into DNA by covalent bonding of the Schiff's base with the 2-amino group of a G-base in the minor groove of DNA. If the interaction with DNA occurs at the trinucleotide 5′-NGC-3′, then the drug intercalates between N and G and covalently bonds to the G-base on one strand using formaldehyde, and to the G-base on the opposing strand using hydrogen bonds. This combination of intercalation, covalent bonding, and hydrogen bonding is referred to as the *virtual cross-linking* of DNA by anthracyclines (Taatjes & Koch, 2001). Overexpression of formaldehyde dehydrogenase decreases the amount of formaldehyde in cells, and this feature is supposed to be one of the mechanisms responsible for resistance to the formation of anthracycline-formaldehyde conjugates. The expression levels of formaldehyde dehydrogenase in doxorubicin-resistant human small-cell lung carcinoma cell lines were actually lower than in sensitive lines (Brazzolotto et al., 2003). Properties of genomic dsDNA isolated from neuroblastoma cells were studied via an adsorptive transfer technique in connection with square wave voltammetry in our ongoing study. A decrease in the cytosine/adenine signal (CA) was significant in DNA isolated from neuroblastoma cells sensitive to anthracyclines cultivated in the presence of 0.1 to 0.5 mM doxorubicin, but not from resistant cells cultivated under the same conditions. The 0.5 mM concentration of doxorubicin, which is ~100 times higher than clinically relevant doses, decreased the CA signal by more than 30% (Huska et al., 2009a, 2009b; Trnkova et al., 2009). This finding suggests that in chemoresistant cells, the levels of anthracyclines covalently bound to DNA are decreased, but there is still a debate as to whether this phenomenon is relevant to clinical practice.

Anthracyclines enter cells via passive diffusion, and intracellular accumulation can result in concentrations that are 10- to 500-fold greater than extracellular levels (Robert & Gianni, 1993). The efficiency of anthracycline uptake depends on their lipophilicity. All anthracyclines are substrates for P-glycoprotein, which is an ATP-binding cassette transporter (ABC transporter) with broad substrate specificity. It likely evolved as a defense mechanism against harmful substances including cytostatics (anthracyclines, etoposide, vinca alcaloids). Idarubicin is a less avid substrate for P-glycoprotein, which may explain its higher efficacy in chemoresistant tumors. In addition, resistance to anthracyclines may be caused by point mutations in a gene of topoisomerase IIα or its down-regulation. It seems, however, that chemotherapy resistance probably requires changes in the expression of a large number of genes (Bedrnicek et al., 2005).

Structural effects determine the rate of drug penetration into the nucleus of a cancer cell, the conformation of the complex formed between the drugs and their target, and the specificity and energetics of drug–DNA interactions. Therefore, a detailed understanding of the contributions of specific structural modifications in an anthracycline molecule to its toxic interactions should constitute an extremely important finding to explain its mode of action. To explore the mechanisms of action of anthracycline antibiotics, studies on the interactions of drugs with DNA are necessary to develop novel analogues with higher antitumor activity.

3.3. Computational studies

Anthracyclines have also been explored with various computational tools. Despite the low number of reports, several interesting analyses have been performed as either individual work or as a complement to experimental results. In the late 1990s, Mariam et al. performed a series of purely computational studies (Mariam & Sawyer, 1996; Sawyer et al., 1996; Mariam & Chantranupong, 1998, 2000; Mariam et al., 1999) where several aspects of anthracyclines were explored. The semiempirical Austin Model 1 (AM1) method was applied to electron transfer reactivity (Sawyer et al., 1996). Adiabatic ionization potentials (IPs) and electron affinities (EAs), as well as some other electronic properties determined for a chosen model system, did not support the concept of the redox activity of 5-

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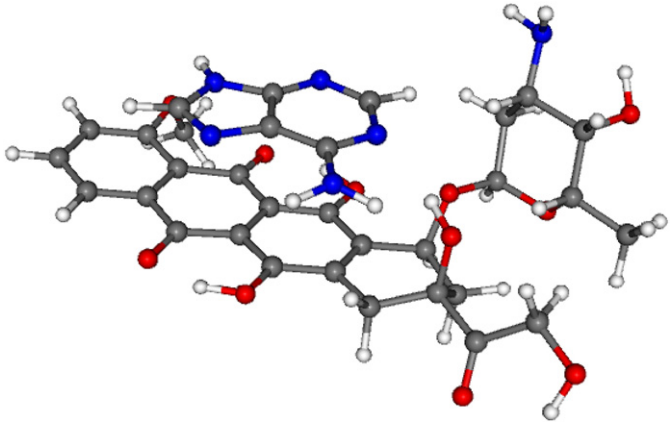

Fig. 5. The most stable structure of the isolated doxorucin-adenine complex optimized at the RI-DFT(BLYP)-D/SVP computational level.

iminodaunomycin differing in comparison to daunomycin. The difference in redox capacity of a two-ring model of aclacinomycin A and naphthacenedione was found to be negligible. In the study, the authors focused on aspects of the reduced toxicity of aclacinomycin A and 5-iminodaunomycin. Another subject mentioned in the study was the influence of the keto-enol tautomer equilibrium and the importance of hydrogen bonding, which play a significant role due to the relationship between electron configuration or density and the geometric parameters. The AM1 method was also used for determination of the adiabatic electron affinities of neutral hydroquinone radicals (Mariam & Sawyer, 1996). Other important phenomena in anthracycline topics include hydrogen-bonding interactions and proton transfer in their tautomeric forms. 1,4-Dihydroxy-5,8-naphthoquinone imine was used as a model for 5-iminodaunomycin. The calculations suggested that the N–H…O hydrogen bonds were stronger than or comparable to the strength of the O–H…O hydrogen bonds. This preference was, however, in contrast with nuclear magnetic resonance (NMR) data of 5-iminodaunomycin (Lown et al., 1979; Tong et al., 1979). The authors stated that the overall accuracy of the HF/6-31G** (Hartree–Fock theory using a medium-sized basis set) and SAM1 (semi ab initio #1) methods for the study of hydrogen bonding in these systems were unsatisfactory. Since only small model systems were explored at mainly semiempirical levels, further verification of the obtained results was necessary. In the next study (Mariam & Chantranupong, 1998), hybrid density functional (B3LYP, Becke–Lee–Yang–Parr) was employed for determination of the adiabatic EA of 1,4-benzoquinone and 1,4-benzoquinone imine. The results were in reasonable accord with experimental data when the basis set was extended by diffuse functions. Later, the DFT method was also used to investigate intramolecular hydrogen-bonding interactions in a model system of 5-iminodaunomycin (Mariam & Chantranupong, 2000). BLYP (Becke–Lee–Yang–Parr) and hybrid B3LYP functionals predicted the relative decreasing order of hydrogen bond strength to be: O–H…N (strong)≥O–H…O (strong)>N–H…O, in contrast to the experimentally observed one: O … H–O (strong)>N … H–O (normal)>O … H–N (weak). Despite the relatively accurate method used in this case, an important feature in obtaining agreement with experimental results was to consider solvent effects. Therefore, a polarizable continuum model (PCM) should be included in such calculations (Zimmermann & Burda, 2009). Cashman and Kellogg(2004) investigated anthracycline binding to specific sequences of DNA. The Hydropathic INTeractions (HINT) program was utilized to describe various binding, including differences in the functional group contributions as well as sequence selectivity (Kellogg et al., 1991). Several compounds have been identified that include features that may enhance sequence selectivity. In addition, removal of the methoxy group at the C-4 position on the aglycone moiety appears to add potency and selectivity (as noted with idarubicin). Trieb et al. explored cooperative effects when daunomycin intercalates into the B-DNA duplex using the AMBER program. The authors estimated the intercalation energy to be up to 32 kcal/mol (Trieb et al., 2004). Dissociation reactions of protonated anthracyclines (epirubicin, daunorubicin, idarubicin) were compared using electrospray ionization with tandem mass spectrometry (Sleno et al., 2006). The absolute proton affinity values were calculated at the B3LYP/6-311+G** level and were used to support the role of selected functional groups of the anthracyclines. Recently, Lu et al. studied the interaction of a daunorubicin with calf thymus DNA. The molecular modeling CHARMM program was used to complement UV–vis and fluorescence spectroscopy to simulate the interaction of anthracycline with dodecamer duplex DNA (Lu et al., 2010). Daunorubicin slides into the C–G rich region of ctDNA with an estimated ΔG of about −42 kJ mol^{-1}.

We also performed a set of gas phase optimizations of the stacking interactions of isolated nucleobases (G, A, C, T) with doxorubicin at the RI-DFT(BLYP)/SVP (Resolution of Identity Density Functional Theory (Becke–Lee–Yang–Parr)/Split-Valence basis sets type P) level (Eichkorn et al., 1995) using the program Orca 2.6. version 35 (Kossmann & Neese, 2009) with empirical corrections for dispersion interactions, as suggested by Grimme(2006). The following single point (SP) calculations were performed with the program Turbomole version 5.9 (Ahlrichs et al., 1989) at the RI-MP2/aug-cc-pvdz (Resolution of Identity-Møller–Plesset-2) level, including BSSE and deformation energy corrections. We found that adenine forms the strongest stacked complex and is further stabilized by H-bonding interactions between N-1 and H-(N^6) with a hydroxy group of the amino sugar daunosamine. The optimized structure is displayed in Fig. 5, and the total stabilization energy was about −49 kcal/mol. A structure similar to guanine exhibited a stabilization energy of −43 kcal/mol. As for the pyrimidine nucleobases, the cytosine stabilization energy was −37 and thymine stabilization was −32 kcal/mol. These stacked structures cannot be directly compared with anthracycline intercalation since a different orientation of nucleobases and anthacycline is enforced in the intercalation complex due to steric conditions, as well as of the presence of different hydrogen bonds since the daunosamine sugar lies in the minor groove. Daunosamine forms (strong) H-bonds at the Watson–Crick pairing edge by interacting with isolated nucleobases. Our data can be indirectly compared with nucleobase stacking interactions, where very accurate estimates exist (Hobza & Sponer, 2002; Zhao & Truhlar, 2005). The A…T stack formation is connected with ~10 kcal/mol, while in G…C and C…C stacking, about 16 and 10 kcal/mol are released. From those results, one could expect larger stacking interactions for guanine. However, upon comparing the mutual dipole moment orientation of adenine and the anthracycline aromatic system together with the H-bonding of N-1 HN^6 to daunosamine, slightly higher interactions would be expected.

4. How to scavenge reactive oxygen species produced by the drugs

Because reactive oxygen species (ROS) are responsible for the toxic side effects of anthracyclines, studies on their scavenging are essential for clinical practice. In addition, one quinoline derivative structurally related to ellipticine [8-methyl-4-(3-diethylaminopropylamino) pyrimido [4′,5′;4,5] thieno (2,3-b) quinoline] mediates the production of ROS (Shenoy et al., 2007). ROS are involved in processes associated with cell growth, differentiation and death (Fig. 6). Low concentrations of ROS are indispensable in the process of cell signaling and defense responses against pathogens, whereas high concentrations of ROS play an important role in the process of aging, ischemia, cancer, immune disorders and endocrine functions (Valko et al., 2006, 2007). Numerous non-enzymatic and enzymatic pathways have been developed as protection against the effects of

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ROS and oxidative stress (Mates, 2000; Ueda et al., 2002). Cellular antioxidant defense is based on redox reactions of amino acid chains, especially cysteine, methionine, tyrosine, phenylamine and tryptophan. Changes in the oxidation state of these molecules are controlled by different redox mechanisms, such as hydroxylation, electron transfer and exchange reactions. In addition to simple amino acid and phenolic compounds, sulfiredoxin (glutathione pathway) and thioredoxin (the reduction of disulfides in proteins) are also involved in the cell redox system. Reduced glutathione (GSH) is the most important cellular antioxidant (Markovic et al., 2010). The GSH/GSSG (oxidized glutathione) ratio can be an excellent indicator of cell redox status. The GSH molecule is closely related to the enzymes glutathione peroxidase, catalase and peroxiredoxin. Glutathione-S-transferase mediates a conjugation of GSH with electrophilic compounds (lipid peroxides and other products of the ROS response). It was found that oxidative stress increases the expression of genes associated with antioxidant proteins. Many antioxidant proteins are expressed without oxidative stress, mainly to regulate the natural homeostasis of ROS in cells. The known redox-regulated transcription factors include NF-κB and transcription activator protein AP-1 (Jia & Misra, 2007; O'Hara et al., 2009). NF-κB activity is induced by the presence of peroxide. The AP-1 protein is a dimer composed of the products of protooncogenes Fos and Jun (both are strongly induced by oxidative stress). In addition, the nuclear protein Ref-1 significantly affects the DNA binding activity of AP-1 (O'Hara et al., 2009).

Aside from systems based on the enzymatic reactions mentioned above, the thiol groups of cysteines are capable of forming coordination bonds with metal ions generating ROS, including iron, zinc, mercury, cobalt, and copper, and can also be included in the antioxidant defense system. This category includes many proteins (zinc fingers, dehydrogenases and others). A prominent member of this system is a protein containing twenty cysteine residues called metallothionein (MT), which constitutes a metal binding domain juxtaposed with basic amino acids (lysine and arginine) arranged in two thiol-rich sites called the α and β sites (Eckschlager et al., 2009; Krizkova et al., 2009; Adam et al., 2010). The cysteine sulfhydryl groups can bind 7 moles of divalent metal ions per mol of MT, while the molar ratio for monovalent metal ions (Cu and Ag) is twelve. Although the naturally occurring protein has Zn^{2+} in the α and β binding sites, this ion may be substituted with another metal ion that has a higher affinity for thiolate such as lead, copper, cadmium, mercury, silver, iron, platinum and/or palladium (Adam et al., 2005; Petrlova et al., 2006; Krizkova et al., 2007; Eckschlager et al., 2009).

It has been shown that various cancers such as kidney, stomach and prostate cancer are characterized by the presence of significant oxidative stress that is induced by a strong proliferative activity of tumor cells. In addition, most anticancer drugs (anthracyclines, cyclophosphamide, platinum derivatives, mitomycin, fluorouracil, and cytarabine) and radiotherapy induce ROS in healthy tissues, which is involved in many of their adverse effects (Maritim et al., 2003; Pelicano et al., 2004). The issue of ROS induction by these drugs has not yet been given significant attention. A possible approach is the protection of healthy tissues to prevent the emergence of ROS, and one promising strategy is to prevent the emergence of ROS. A number of strategies have been suggested for reducing the toxic effects of chemotherapy, mainly cardiotoxicity and neurotoxicity. These include the gradual administration of drugs and the use of analogues or modification of the drug. A possible approach is the protection of healthy tissue to prevent the emergence of ROS. One strategy is to interrupt the chain of ROS. Dexrazoxane is an iron-binding chelator used with anthracyclines to reduce the levels of ROS, but because of the potential risks with other drugs and the lack of knowledge of the corresponding interactions, the use of dexrazoxane in cancer patients is not widely recommended (Jones, 2008). Similarly, the use of TNF-α has been associated with neurotoxicity caused by an enhancement of nitric oxide synthase activity in the brain, which can be inhibited. Other proteins, such as manganese superoxide dismutase (MnSOD), catalase, MT, a thioredoxin, can be applied to significantly inhibit oxidative stress by scavenging radicals via free –SH moieties. MT can serve as a "maintainer" of the redox pool. ROS enter through the cytoplasmic membrane of the cell. MT synthesis is increased via activation of metal-regulatory transcription factor-1 (MTF-1), which interacts with metal ions (Fig. 6). Biological disulfides such as glutathione disulfide (GSSG) oxidize MTs with a concomitant release of zinc, while glutathione (GSH) reduces the oxidized protein to thionein, which then binds to available zinc. The GSH/GSSG redox pair can be efficiently coupled with MTs. This coupling could provide a very effective tool to modulate oxidation and reduction (Maret & Vallee, 1998). Liu et al.(2008) reported on the release of Zn from MTs by intracellular oxidants such as GSSG. Moreover, nuclear MTs can protect cells against UV and ionic radiation (Reeve et al., 2000), as well as against some cytotoxic alkylating agents, including chemotherapeutics (Okazaki et al., 1998; Sunada et al., 2005). MTs stabilize lysosomes and decrease apoptosis following oxidative stress through the inhibition of Fenton-type reactions and by ensuing peroxidation of lysosomal membranes (Baird et al., 2006). The mechanism involves an antioxidant response element (ARE) in the promoter region, ARE binding transcription factors, MTF-1, transcription factors of the basic zipper type (Fos and Fra-1), NF-E2-related factor 2, and the upstream stimulatory factor family (USF, a basic helix–loop–helix–leucine zipper protein), although it is probable that other unidentified proteins are involved in these mechanisms (Haq et al., 2003). The synthesis of MT can also be triggered by the presence of ROS (Fig. 6).

Aside from scavenging ROS by the processes mentioned above, another way to decrease the toxicity of the anticancer drugs is through the administration of antioxidants or their precursors, which can reduce oxidative stress and significantly reduce damage to non-target tissues. Some natural substances like grapefruit juice and grape seed extract have decreased anthracycline side effects in experimental animals (Yalcin et al., 2010). Flavonoids play an important role, because these compounds are modulators of transport molecules in those protective effects (Aszalos, 2008). Similarly, the use of ethyl ester gamma-glutamyl-cysteine as a precursor of GSH increased the GSH content in the brain and enhanced the activity of glutathione-S-transferase, leading to a decrease in ROS burden in mice (Joshi et al., 2007). The use of these substances shows their potential to protect healthy tissues in cancer chemotherapy.

5. Nanocarriers as a new tool in the targeting of ellipticines and anthracyclines

Nanomedicine, one of the newest branches in medicine, is defined as the monitoring, repairing, building, and control of biological systems, as carried out by nanocomponents and nanosystems. It has enabled the incorporation of drugs into nanoparticles ranging from 10 nm to 1 μm, which improve the therapeutic possibilities in oncology and allow drug release based on changes in the extracellular matrix or the affinity of nanoparticles for the walls of cancer cells (Jain, 2010). Over 20 nanoparticle-delivered drugs have been registered by the FDA for clinical use, e.g., liposomes (pegylated liposomal doxorubicin and liposomal daunorubicin), albumin-bound paclitaxel, and polymeric particles (PLA/MPEG-PLA paclitaxel) (Jain & Stylianopoulos, 2010). Other doxorubicin nanodrugs are in phase I clinical studies, like dextran-doxorubicin for the therapy of various cancers and PEG-aspartic acid doxorubicin micelles for pancreatic cancer, and phase II, including HPMA copolymer doxorubicin in two studies, one for lung and breast cancer and the second for hepatocellular carcinoma (Ali et al., 2011). The development of each new drug has two main aspects — maximal effectiveness against the disease and minimal side effects. The nanoparticle-mediated targeted delivery of drugs might significantly reduce the dosage, increase its specificity and bioavailability and reduce toxicity (Gu et al., 2007; Chomoucka et al., 2010; Patra et al., 2010).

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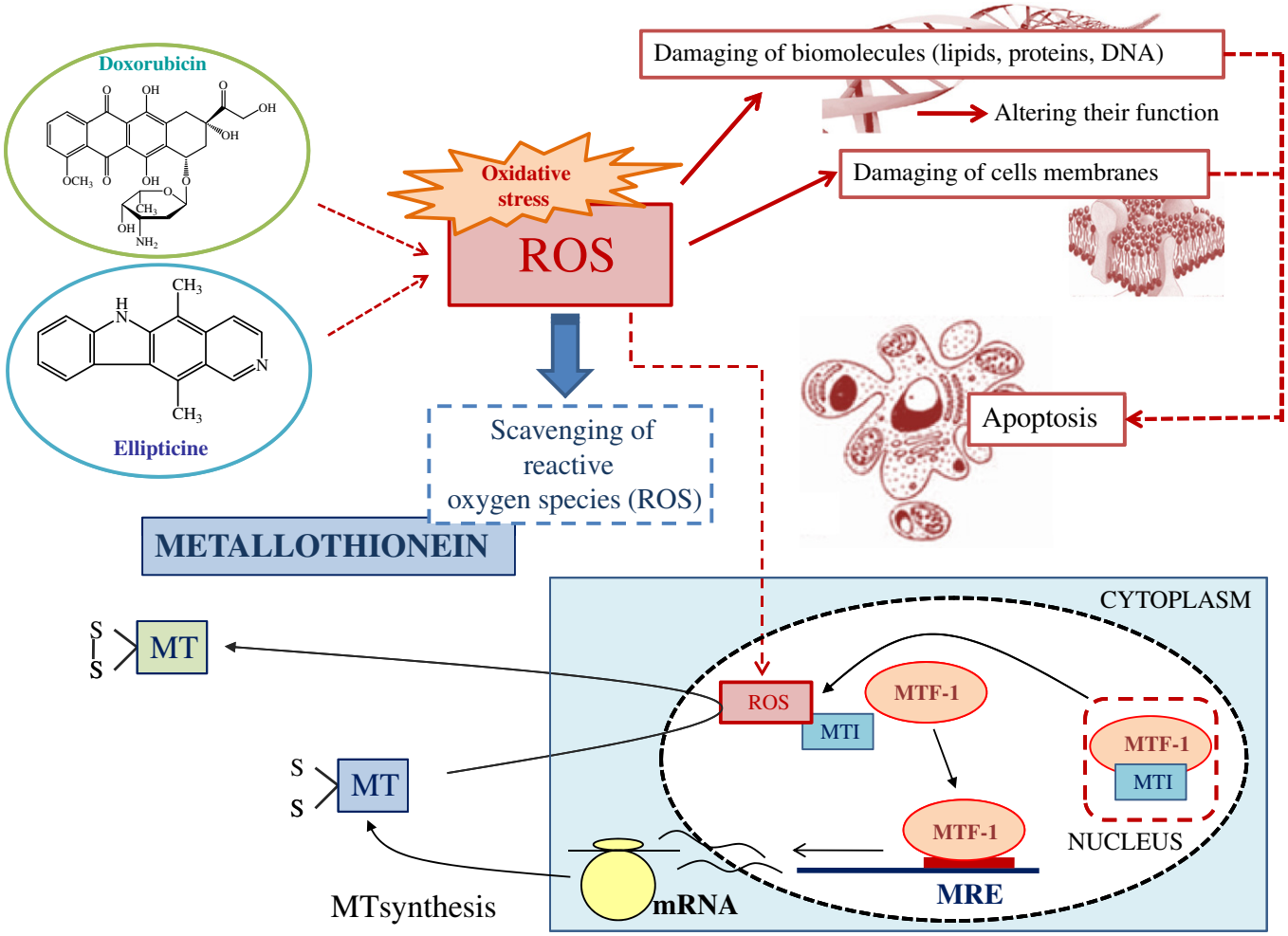

Fig. 6. Scheme of possible interactions between doxorubicin and ellipticine with metallothioneins. Doxorubicin and ellipticine derivatives can generate reactive oxygen species (ROS). These species can damage cell membranes and biomolecules, which can, in turn, induce apoptosis. One-electron addition to the quinone moiety in ring C of DOX and other anthracyclines has long been known to result in the formation of a semiquinone that quickly regenerates its parent quinone by reducing oxygen to ROS, such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). This futile cycle is supported by a number of NAD(P)H-oxidoreductases (cytochrome P450 or -b_5 reductases, mitochondrial NADH dehydrogenase, xanthine dehydrogenase, and endothelial nitric oxide synthase (reductase domain)) (Minotti et al., 2004). Damage of the membranes and biomolecules can interfere in numerous crucial biochemical pathways. However, a cell has pathways to scavenge ROS; the synthesis of metallothioneins as scavengers can be considered as one of them. ROS enter through a cytoplasmic membrane of a cell. After entering the cytoplasm, the ions can interact with a complex of metal-regulatory transcription factor-1 (MTF-1) and metal synthesis inhibitor (MTI). The ions bind to MTI. Then, MTF-1 is released and can bind to a regulatory sequence of DNA called the metal responsive element (MRE). Thereafter, the gene responsible for the synthesis of metallothioneins is transcribed. The synthesized mRNA molecule is translated into MT, which scavenges ROS.

Tumor targeting with nanoparticles that can be modified with various types of materials, including biomolecules, can be realized using passive and active methods (Palecek & Fojta, 2007; Drbohlavova et al., 2009; Chomoucka et al., 2010).

The first mentioned is based on the enhanced permeability and retention effect of the tumor vasculature. The active way relies on ligand-directed binding of nanoparticles to receptors expressed by tumor cells (Wang et al., 2010). The key features of anticancer nanoparticles are mainly their size, surface properties and targeting ligands. Nanoparticles designed for tumor-targeted therapies consist of various components, and in most cases are based on nanocarriers composed of iron oxides, gold, biodegradable polymers, dendrimers, lipid based carriers such as viruses (viral nanoparticles) or organometallic compounds and an active agent (drug) (Ferrari, 2005; Mishra et al., 2010). Drug-carrier nanoparticles are considered to be systems that act as drug vehicles, as either nanospheres (a matrix system in which the drug is dispersed) or nanocapsules (reservoirs in which the drug is confined in a hydrophobic or hydrophilic core surrounded by a polymeric membrane) (Juillerat-Jeanneret, 2008). Drug encapsulation in a nanocarrier provides better biocompatibility, and hence, its potential use in clinical oncology.

The following materials and strategies are of interest for chemists and clinicians as potential delivery systems for drugs: polymeric nanocarriers, liposomes, micelles, polyethylene glycol, poly(lactic-co-glycolic acid), dendrimers, hydrogel, and nanoparticles (gold, silica and magnetic nanoparticles, quantum dots, and carbon nanotubes). Some important characteristics of liposomal cocktails involving the combination of two cytotoxic drugs have been recently reviewed by Chiu et al.(2009).

Liposomes have been used in numerous papers as successful anticancer drug carriers (Al-Jamal et al., 2008; Il Kang et al., 2009; Narayanan et al., 2009; Matsui et al., 2010; Wenzel et al., 2010). Several such engineered drugs are already in clinical practice, including liposomal doxorubicin, which is less cardiotoxic than unencapsulated doxorubicin (Haley & Frenkel, 2008). Liposomal delivery systems are the superior method to passively target anthracyclines to tumors. It has been proven in a randomized clinical trial that liposomal doxorubicin is favored over conventional administration. Pegylated liposomal doxorubicin has better pharmacokinetic properties than doxorubicin. It induces fewer side effects, as administration at a minimum cumulative dosage from 500 to 1500 mg/m^2 did not cause congestive heart failure, while safe cumulative

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doses of conventional doxorubicin are 350–400 mg/m^2 (Carvalho et al., 2009; Puri et al., 2009).

The blood–CNS barrier that protects brain tumors from several cytostatics is permeable to lipid-insoluble macromolecules. In preliminary pre-clinical studies, it has recently been shown that one intravenous dose of 7–10 nm particles with doxorubicin bound to the particle exterior via acid-labile covalent linkages is effective at regressing orthotopic rodent malignant glioblastomas (Sarin, 2010).

Liposomal cocktails of two cytostatics have also been tested (Lim et al., 2010). The efficacies of a liposome cocktail of cytarabine and daunorubicin (the cytostatics used in the therapy of acute leukemia) in a molar ratio of 5:1 displayed the greatest degree of synergy and minimum antagonism, and were more efficient than the saline-based cocktails in the therapy of acute leukemia in mice (Tardi et al., 2009).

6. Conclusion and future perspectives

The data shown in this review indicate high efficiencies of drugs based on doxorubicin and ellipticine in cancer treatment. The toxic side effects of doxorubicin and its analogue (anthracyclines), such as cardiotoxicity mediated by ROS, are a liability and processes that protect against such effects are suggested in this review. Nevertheless, novel strategies based on recent findings in the field of nanotechnologies for safer drug delivery and those in the field of gene therapy for the blocking of repair mechanisms need to be explored to improve the anticancer action of these two classes of cytostatics and decrease their adverse effects. One of the most frequently used anticancer drugs in the form of different carriers is doxorubicin. Its toxic effects have been successfully reduced by employing various nanoparticle types as drug carriers, such as micelles, polymer-based nanoparticles, liposomes, and magnetic particles. Likewise, nanoparticles (micelles) have been found to be promising carriers for ellipticine. In addition, ellipticine should be a suitable candidate for CYP- and peroxidase-gene-directed enzyme-prodrug therapy (Lu et al., 2009), which has the potential to provide efficient activation of ellipticine in target tumor tissue.

In addition, nanocarriers were described that have been shown to have ABC transporter inhibitory activity. There are various mechanisms that can be responsible for efflux pump inhibition, e.g., membrane fluidization, ATP depletion, and interactions with drug-binding or the ATP binding site (Bansal et al., 2009). Water-soluble polymers based on N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are used as a drug carriers because their biophysical and biochemical characteristics are favorable. It has been shown in both animal experiments and clinical studies that HPMA copolymer-bound doxorubicin conjugates have not only anti-tumor, but also immunomodulatory effects and decreased side effects, including myelotoxicity and cardiotoxicity (Rihova & Kovar, 2010). The anticancer specificity and efficacy of HPMA copolymer-bound drugs could be increased by active targeting where antibodies, carbohydrates and lectins that may serve as ligands for receptors on the target cell are linked (Rihova, 2009).

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