0026-895X/97/050882-04$3.00/0

MOLECULAR PHARMACOLOGY, **52:**882–885 (1997).

Epirubicin-Induced Oxidative DNA Damage and Evidence for Its Repair in Lymphocytes of Cancer Patients Who Are Undergoing Chemotherapy

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Received October 18, 1996; Accepted August 5, 1997

SUMMARY

Anthracycline derivatives have been widely used in the treatment of several types of human malignancies. Cytotoxicity of these drugs has been attributed to inhibition of topoisomerase II as well as intracellular production of free radicals. In our work we used a gas chromatography/mass spectrometry technique to study free radical-induced DNA base modifications in chromatin isolated from lymphocytes of cancer patients who received chemotherapy with epirubicin (one of anthracycline's antitumor derivatives). The anticancer therapy caused significant increases in the amount of all four DNA base modifications over control levels in the lymphocytes of most of the patients. For the majority of the cases the base products returned to the control value 24 hr after the infusion of the drug, which suggests the removal of these lesions by cellular repair processes. However, some of the modified bases escaped repair. Because part of these modifications may possess premutagenic properties, they may be responsible for secondary cancers induced by chemotherapy.

Different anthracycline derivatives have been used successfully in the treatment of a wide spectrum of neoplasias for over two decades (1). Intracellular production of free radicals along with intercalation with DNA and subsequent inhibition of topoisomerase II is generally accepted as the major mechanism of anthracycline cytotoxicity (2). Anthracycline-produced free radicals can be generated by both enzymatic and nonenzymatic mechanisms (3–5). Free radicals may cause damage to biological molecules including DNA. They can produce different kinds of DNA lesions, among them free radical-modified DNA bases. Base damage appears to be an important class of lesions because some of them may possess mutagenic properties and may lead to carcinogenesis (6–9). Thus the observed mutagenicity of anthracyclines may be, at least in part, attributed to the generation of reactive oxygen species (2). These changes may also be responsible for the formation of secondary cancers in patients undergoing chemotherapy.

Recently, using a GC-MS method, Akman *et al.* (10) have shown that reactive oxygen reduction by the redox cycling of the doxorubicin quinone moiety is responsible for DNA base modification in isolated human chromatin. To address the question of whether a similar phenomenon can occur *in vivo* we used a GC/MS technique to analyze chromatin isolated from lymphocytes of cancer patients undergoing chemotherapy. We also wanted to determine whether removal (repair) of the base damage can be observed in lymphocytes after completion of chemotherapy.

These studies were supported in part by grants from the Polish State Committee for Scientific Research (KBN), Grant 4PO5A.121.08, and from the USA-Polish Maria Sklodowska-Curie Joint Fund II.

Materials and Methods

Chemicals

Modified DNA bases, their stable isotope-labeled analogues, thymine-$\alpha,\alpha,\alpha,6$-$^2H_4$, and materials for GS/isotope-dilution MS were obtained as described previously (11).

Patients. Blood samples were obtained from 14 cancer patients undergoing chemotherapy at the Provincial Center of Oncology, Bydgoszcz, Poland. The patients were from 35 to 51 years old. They received 70 mg of epirubicin/m^2 (Farmitalia Carbo Erba, Milan, Italy) in a single injection.

Patients 1–5 and 10–14 suffered from different form of sarcomas, patient 6 and 7 suffered from ovarian cancer, patient 8 suffered from breast cancer, and patient 9 from thyroid gland cancer. All the patients except patients 1 and 7 received chemotherapy for the first time. Patient 1 had been exposed to cyclophosphamide, 5-fluorouracil, and methotrexate 1 year before the treatment, whereas patient 7 received cisplatin, vinblastine, and bleomycin 2 years before the treatment.

Blood collection and isolation of chromatin. Venous blood samples (15 ml) were drawn from the patients before the treatment (control sample), 1 hr after the infusion of the drug, and 24 hr after

ABBREVIATIONS: GC, gas chromatography; MS, mass spectrometry.

the infusion. Samples were collected in EDTA-coated tubes. Each blood sample was divided into three aliquots. The aliquots of blood were carefully applied on the top of a Gradisol L solution (Polfa, Kutno, Poland), and the lymphocytes were isolated according to the procedure supplied by the manufacturer. Microscopic evaluation showed that 95% of the cells were lymphocytes. Chromatin from lymphocytes was isolated and evaluated as described previously (12). Three independently isolated chromatin samples were obtained from each blood sample.

Analysis by GC/MS. Aliquots of stable isotope labeled internal standards were added to the chromatin samples containing 100 μg of DNA (11). After hydrolysis, the DNA bases were converted into volatile derivatives; then, the samples were analyzed by GC/isotope-dilution MS with selected ion-monitoring (11). Thymine-$\alpha,\alpha,\alpha,6$-$^2H_4$ was used as an internal standard for thymine to verify the amount of DNA in the chromatin samples (13).

Student's t test from the Statistica 4.5 program was used for statistical analysis. A difference was considered to be statistically significant when $p < 0.05$.

Results

Using GC/MS, we measured the endogenous amounts of oxidatively modified DNA bases (control samples) as well as the level of these modifications in chromatin after chemotherapy was applied. Chromatin was isolated from lymphocytes of cancer patients undergoing chemotherapy with epirubicin. (Clinical use of the drug was described in Ref. 14.) The following base products were identified and quantified in cellular DNA: 8-hydroxyguanine, 8-hydroxyadenine, 2-hydroxyadenine, 5-hydroxyuracil, 5-hydroxycytosine, and thymine glycol.

There were considerable interindividual differences in the amount of modified bases in untreated samples as well as in the samples isolated after the drug infusion (Fig. 1). Similar variation of the base levels were observed by us (15–17) and others (18) in DNA isolated from lymphocytes as well as from cancerous and noncancerous tissues of patients suffering from different types of cancer.

Significant increases in the amounts of modified bases over the control level were observed in the samples isolated 1 hr after the infusion of the drug in examined patients. (No response to chemotherapy regarding the increases of the base products was observed in lymphocytes of one patient. For most of the patients and most of the modified bases, the amount of products decreased 24 hr after infusion of the drug, in most cases reaching control levels. Statistically significant changes (for all base products) were observed for the entire patient group (Fig. 1). In the case of two base products (ThyGly and 2-hydroxyadenine), after an initial increase the level of these modified bases remained high 24 hr after the treatment.

Efficacy of therapy. Patients 1, 3, 5, 7, 12, and 13 died 4–12 months after completion of therapy. In the case of patients 9 and 10, remissions were observed. We did not find any changes in cancer development during a 1-year period for patients 2, 8, 11, and 14, whereas in the case of patients 4 and 6, progression of the disease was observed.

Discussion

In recent work Akman *et al.* (10) have shown that in isolated chromatin biologically relevant doses of doxorubicin introduced DNA base damage typical for −OH radical attack. In the present work using epirubicin (chemical name: 4-epidoxorubicin, the analog of adriamycin presenting a different configuration of the −OH group in the C-4 position of the amino sugar moiety; Fig. 2), we observed a similar phenomenon in chromatin isolated from lymphocytes of cancer patients undergoing chemotherapy. The pattern of these modifications also suggests the involvement of the −OH radical in their formation.

Recently, using the EPR technique and different anthraquinone complexes, it has been shown that both anthracyclines, adriamycin and epirubicin, bound to Fe(III), can produce −OH radicals by enzymatic and nonenzymatic mechanism (19). In this context it is noteworthy that chemotherapy can be responsible for the rise in serum iron (20, 21). There is also a possibility that iron can be present in a nucleus (22). Because anthracycline derivatives can intercalate within the DNA molecule, the condition exists for the formation of the −OH radical in close vicinity to the DNA and thus for base modifications.

The extent of the base modifications differs among patients. This individual variability may reflect individual dif-

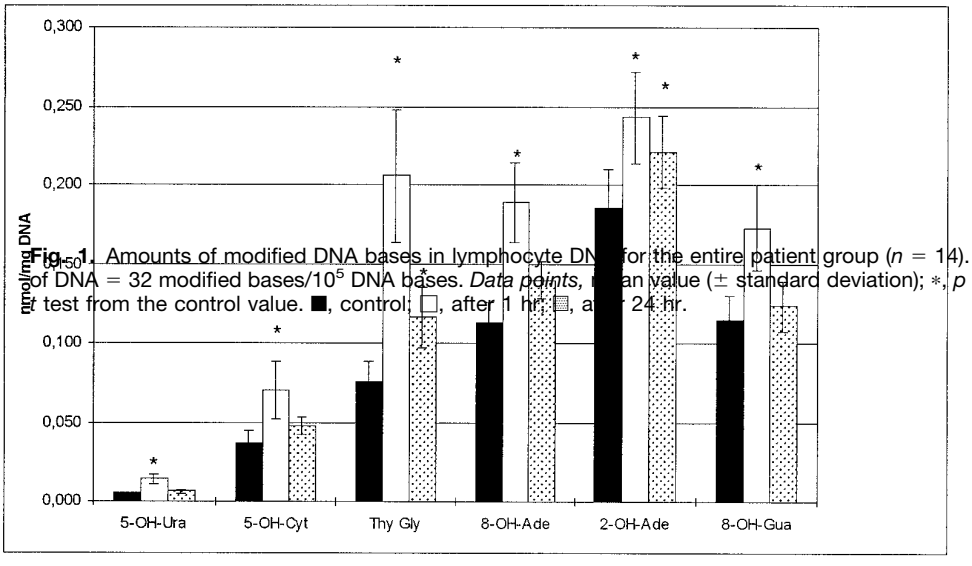

Fig. 1. Amounts of modified DNA bases in lymphocyte DNA for the entire patient group ($n = 14$). One nanomole of modified DNA base/mg of DNA = 32 modified bases/10^5 DNA bases. *Data points,* mean value (± standard deviation); ∗, $p \leq 0.05$, significantly different by Student's t test from the control value. ■, control; □, after 1 hr; ▨, after 24 hr.

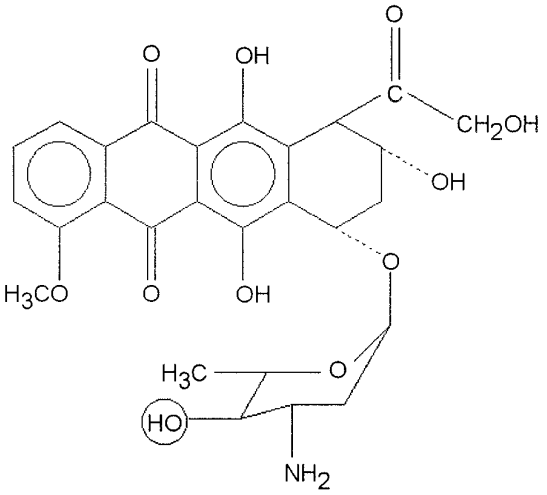

Fig. 2. Chemical structure of epirubicin (4′-epiadriamycin): the −OH group in C-4′ position presents the equatorial configuration.

ferences in metabolism and repair capacity and, at least in part, genetic background (23, 24).

An indirect proof for the formation and removal of one modified base has come from the recent work of Faure *et al.* (25). They reported that urine HMUra is significantly increased in the Adriamycin-treated patients. Because oxidized bases are poor substrates for the enzymes involved in nucleotide synthesis, they are generally excreted into urine and may serve as biomarkers of the DNA repair process (26–28). Therefore, the above quoted results suggest the removal of one of the base products, in good agreement with our study. However, in our study we directly demonstrated formation of the base modification products derived from all four bases. Also, as the authors of this quoted article (25) admit, in their study patients received Adriamycin therapy in association with other drugs that can also be involved in the production of free radicals, whereas in our study patients received solely epirubicin.

To our best knowledge, the results of the present study reveal for the first time, not only the modifications of all four DNA bases, but also removal (repair) of these products from the lymphocyte DNA of cancer patients undergoing chemotherapy. For most of the patients and most of the base products, 24 hr after the infusion of the drug the level of modified bases returned to the control value.

Several enzymes have been identified that can specifically recognize and remove modified DNA bases (29). Moreover, just recently it has been demonstrated that the base modifications analyzed in this work are the subject of repair processes in human lymphoblast cells *in vitro* (30). Usually after 4 hr all damaged bases were removed from cellular DNA (30). However, in our study, in the case of some patients and some modifications the level of damage remained elevated 24 hr after their introduction. This suggests that in human lymphocytes *in vivo* some of the lesions escape the repair process.

There is a risk of developing secondary cancers after chemotherapy (31). Long-lived B and T lymphocytes may serve as a target cells for carcinogens including some anticancer drugs (32). Some base modifications that escape repair in lymphocyte DNA could lead to mutagenesis in critical genes and ultimately to secondary cancers. This notion is supported by the fact that the treatment of laboratory animals with carcinogenic agents generates similar modifications in the genomic DNA of the target organs of animals before tumor formation (33–35). Moreover, the relevance of oxidative DNA base damage to development of secondary cancers stems from the premutagenic properties of some of the DNA lesions observed in this study. Thus, 8-hydroxyguanine causes GC-to-TA transversion (36–38). 8-hydroxyadenine also possesses premutagenic properties (39). 5-hydroxycytosine and 5-hydroxyuracil have been shown to be a potentially premutagenic lesion leading to GC-to-AT transitions and GC-to-CG transversions. 5-Hydroxycytosine seems to be more mutagenic than any other product of oxidative DNA damage (9).

Patients differ in their responses to chemotherapy. As has been demonstrated above there are also individual differences with respect to the formation as well as removal of the base products after infusion of the anticancer drug. Therefore, the possibility exists that this variability may partially account for the differences in clinical response to the chemotherapy.

Interestingly in the case of two patients in whom remission was observed, the amount of ThyGly stayed high 24 hr after infusion of the drug. Furthermore, statistical analysis of the entire patient group revealed that ThyGly is the base that stayed high 24 hr after the drug infusion. It is noteworthy that the presence of ThyGly blocked DNA replication (29). Although we do not have enough data to draw definite conclusions, we can hypothesize that induction of ThyGly in DNA of cancerous cells may be one of the reasons for the antineoplastic properties of anthracycline derivatives. Follow-up studies of the surviving patients should allow us to relate critical molecular events, such as formation of modified bases and their repair, to therapy-induced toxicity.

Acknowledgment

The authors are very grateful to Dr. Miral Dizdaroglu (Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, MD) for helpful discussion and for the kind gift of labeled internal standards used in this work.

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