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Increased proapoptotic activity of electron beam irradiated doxorubicin and epirubicin in multidrug-resistant human leukemic cells

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ABSTRACT

This study evaluated the effect of electron beam irradiation on the cytotoxic activity of anthracycline antibiotics such as doxorubicin (DOX), epirubicin (EPI), and dunorubicin (DAU) in human acute lymphoblastic leukemia cell line CCRF-CEM and its multidrug-resistant variant CCRF-VCR1000 cell line characterized by the overexpression of *ABCB1* gene. Drugs were irradiated at doses of 10 and 25 kGy. Data from EPR studies proved that the highest concentration of free radicals was found in DOX and that the number of stable free radicals is always greater after irradiation. In *in vitro* studies, a higher cytotoxic activity of irradiated DOX and EPI in multidrug-resistant CCRF-VCR1000 cells was observed. This tendency was maintained during the storage at 4 °C for 90 days. Changes in CCRF-CEM cells' viability were not dependent on the irradiation status and its dose and were only drug-concentration dependent in all measurement time points. It was proved that increased potency of 25 kGy e-beam irradiated drugs results from their enhanced proapoptotic activity. Apoptotic cell death observed in CCRF-VCR1000 cells treated with irradiated drugs was caspase-8, -9, and -3 dependent and related to the increased Bax/Bcl-2 ratio. No significant differences in the effects of irradiated and non-irradiated drugs on p53 and NFκB transcription factor level and their translocation to the nucleus were noted. Increased activity of the irradiated drugs was not dependent on ABCB1 level.

1. Introduction

The first attempt at radiation sterilization of food products dates back to the beginning of the 20th century [1]. In 1921, the results of the study on the inactivation of *Trichinella spiralis* in pork by exposing the meat to Roentgen radiation were published by Schwartz [2]. In the 1950s and 1960s, governments of USA and Canada approved the use of ionizing radiation for the decontamination of potatoes, crops, and spices [1,3]. In 1980, the expert committees of FAO/IAEA/WHO issued a statement that food products irradiated with doses ≥10 kGy can be consumed with no harm to human health [1,3,4]. So far, a dose of 25 kGy was recommended as sufficient for sterilization of medical products and drugs (Sterility Assurance Level SAL 10^{-6}), and this method has been recommended by pharmacopeias of different countries [5–8]. The method has not only many advantages but also drawbacks of which the most important is the possibility of damage of drug molecules by irradiation. Because of the well-known destructive effect of ionizing irradiation on water molecules, leading to the formation of reactive oxygen species, sterilization by irradiation is applied only to substances in solid state. Although it is estimated that 90% of the drugs can be safely subjected to radiation sterilization, it should always be checked if irradiation would not produce changes in physicochemical properties of a given drug, changes in the drug content, or the appearance of the decomposition of the products above a certain pharmacopoeial norm, also the toxicity of such products should be characterized [8].

Abbreviations: DAU, daunorubicin; DOX, doxorubicin; EPI, epirubicin; IR, irradiated; NIR, non-irradiated; MDR, multidrug resistance.

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Three anthracycline antibiotics such as daunorubicin (DAU), doxorubicin (DOX), and epidoxorubicin (EPI) in the form of hydrochlorides (Fig. 1) have been in the preliminary studies found resistant to ionizing radiation applied in the form of high-energy electrons, in the doses of 10 and 25 kGy [9].

No significant decrease in the content of the above compounds and no changes in their spectral properties were found (FTIR and UV spectra). The appearance of free radicals in the irradiated samples is a natural effect of ionizing radiation and has been observed in many different groups of drugs [8–14]. In the samples of DOX, EPI, and DAU irradiated with a dose of 25 kGy, the presence of free radicals in the concentration of 3.94×10^{15}, 2.44×10^{15}, and 1.37×10^{15} spins/g, respectively, has been detected by EPR method [9]. Varshney and Dodke [10] have reported a three times higher content of free radicals in irradiated DOX, of 1.2×10^{16} spins/g, upon application of a different source of irradiation. There are no norms of the content and structure of free radicals generated on irradiation, but it is obvious that free radicals with unpaired electrons can have a significant effect on the therapeutic activity of medical drugs. That is why the lifetime of particular free radicals should be established, and some authors have even suggested a waiting period after radiation sterilization [8].

Particularly interesting is the influence of free radicals on the pharmacological effects of anthracycline antibiotics, as these drugs, besides alteration of the activity of topoisomerase II and interaction with DNA through the intercalation complexes, also generate free radicals and show peroxidation properties toward lipids and can change the structure of cell membranes [15–17].

The aim of this study was to analyze and identify the free radicals generated by irradiation of the above-mentioned three antibiotics, whose therapeutic activity was determined before and after the irradiation. In the *in vitro* studies, the cytotoxic effect of irradiated and non-irradiated anthracyclines on drug-sensitive (CCRF-CEM) and drug-resistant (CCRF-VCR1000) human leukemic cells was evaluated. In addition, the activity of these compounds after a few weeks (quenching of free radicals) was tested and the obtained data were compared with data acquired directly after the irradiation.

2. Methods

2.1. Drugs

DAU, DOX, and EPI in the form of hydrochlorides (Fig. 1) were synthesized at the Institute of Biotechnology and Antibiotics, Department of Modified Antibiotics, Warsaw, Poland. They were reddish powders, freely soluble in water and methanol.

2.2. Drug irradiation

Of about 0.05 g samples of each substance were placed in 3 ml colorless glass vials that were closed with plastic stoppers and irradiated to 10 and 25 kGy with the e-beam from a linear electron accelerator Elektronika 10/10. The energy of electrons was 9.96 MeV and the current intensity 6.2 μA.

2.3. EPR spectroscopy

Continuous-wave X-band (9.4 GHz) electron paramagnetic resonance measurements were performed on a Bruker ELEXSYS E500 spectrometer with SuperX ER49X Microwave Bridge equipped with Super High Sensitivity Probehead (Bruker BioSpin GmbH, Rheinstetten, Germany). Magnetic field measurements were achieved by an NMT-Teslameter ER 036TM (Bruker BioSpin GmbH, Rheinstetten, Germany). All EPR experiments were performed at room temperature. Spin concentration was obtained after double integration of EPR spectra according to the procedure described elsewhere [18]. The number of free radicals was calculated from EPR spectra recorded at low microwave power (0.5 mW) to avoid saturation effects.

2.4. Cell culture

Experimental model comprising two human leukemia cell lines was used. These lines were the human acute lymphoblastic leukemia cell line CCRF-CEM (ATCC®: CCL-119™) and its multidrug-resistant derivative line CCRF-VCR1000 overexpressing *ABCB1* gene. The cells were maintained in RPMI 1640 culture medium supplemented with 10% fetal bovine serum. The MDR cells were selected by subculturing wild-type CCRF-CEM cells in stepwise increased concentration of vincristine sulfate as described elsewhere [19]. Using PCR method, the cells were routinely tested for the mycoplasma contamination.

2.5. Cell viability assay (MTT test)

The MTT assay was performed, in order to evaluate the cytotoxicity of the tested materials. The cells were exposed for 24, 48, and 72 h to electron beam irradiated (IR) and non-irradiated (NIR) DOX, EPI, and daunorubicin (DAU) in a concentration range of 0.5 nM–1 μM (CCRF-CEM cells) and 0.25–50 μM (CCRF-VCR1000 cells). Subsequently, 20 μl of a solution of MTT (5 mg/ml thiazolyl blue Tetrazolium Bromide; Sigma) were applied. Samples were then incubated for 4 h under standard conditions. Finally, the formazan crystals were released from the cells by adding 100 μl of a solubilizing solution. After the overnight incubation, the absorbance of solutions was measured using a microtiter reader (Multiscan, Labsystems) at two wavelengths: $\lambda = 570$ and $\lambda = 690$ nm.

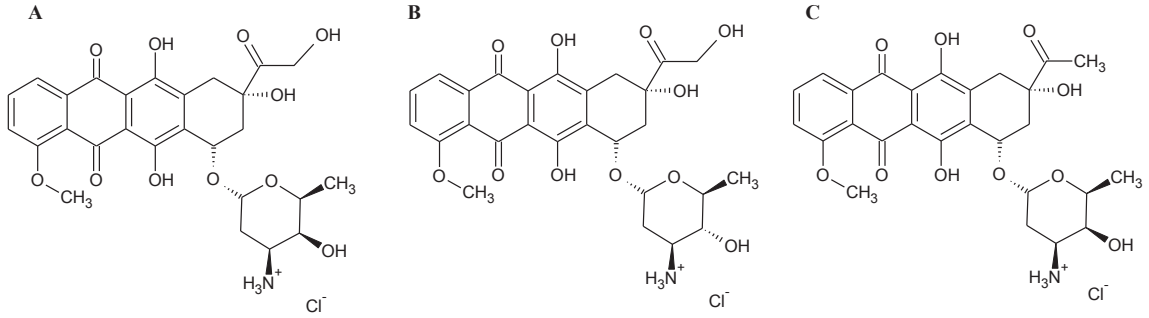

Fig. 1. Structures of anthracycline antibiotics: DOX (A); EPI (B), DAU (C).