The effect of new anthracycline derivatives on the induction of apoptotic processes in human neoplastic cells

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Abstract: This study was an attempt to determine the effect of a selected anthracycline derivative, WP903, on apoptotic processes in human melanoma cells depending on intracellular concentrations of the compound, and to evaluate the significance of apoptosis induction for the cytotoxic effect of anthracycline antibiotics. It was found that the WP903, contrary to ADR (adriamycin) is a strong inducer of apoptotic processes in ME18 human melanoma cells regardless of their susceptibility to adriamycin and WP903. The cells were treated for 24 h with ADR (1 and 5 µg/ml) or WP903 (0.2 and 2 µg/ml). Apoptosis was detected with the use of annexin V-FITC and PI (propidium iodide) and with TUNEL assay. WP903 used at 0.2 µg/ml induced early apoptosis in 23% of ME18 cells and in 60% of ME18/R cells; at 2 µg/ml in 70% of each of cell line tested. Significant late apoptotic effect was observed in ME18 cells. In contrast, ADR was found to be a weak inducer of apoptotic events. The results suggest that apoptosis is not a mechanism directly related to the cytotoxic effect of anthracycline antibiotics.

Key words: Anthracycline derivatives - Apoptosis - Neoplastic cells - Adriamycin - Drug resistance

Introduction

The use of anthracyclines as antitumor drugs dates back to the seventies. In this group of chemotherapeutics, ADR remains constantly the compound that has the widest spectrum of activity. It is used in the treatment of solid tumours, including tumours of the breast, bile ducts, uterus, oesophagus, liver, osteosarcomas and soft tissue sarcomas, as well as acute myeloblastic and lymphoblastic leukaemia [7].

The cytotoxic effect of anthracycline antibiotics is multidirectional. One of the mechanisms consists in blocking the cleavable complex of DNA and topoisomerase II supervising the superhelicity of DNA strands. A positive correlation was found between the cytotoxic effect and topoisomerase II-mediated DNA breaks [2, 7]. The other mechanisms of ADR effect on the cell include formation of adducts (leading, among others, to the formation of crosslinks); intercalation of the ADR molecule between the DNA strands and induction of micronuclei [3, 4, 6]. ADR generates numerous damages in the DNA, and also in cell membrane structure or cell enzymatic systems, due to the free radical mechanism

resulting from metabolic transformation of the drug to a transient radical from [6, 8, 14]. A very important target site for anthracycline antibiotics are cell proliferation control systems including the apoptotic process which is emphasized in the recent years. Ling *et al.* [11] suggest that induction of apoptosis is the primary mechanism of cytotoxic activity of anthracyclines used in the concentration range up to 10 µM. The effect of this group of compounds as apoptosis stimulators on tumour cells is indirect, *i.e.* they influence the activity of specific genes and their products - regulatory proteins.

Because of a serious problem in chemotherapy, and in particular anthracycline therapy, consisting in the phenomenon of multidrug resistance and high cardiotoxicity, new anthracycline derivatives having better therapeutic index are being sought. The search is aimed at selecting derivatives with unchanged or increased antitumor activity, it should lead to cardiotoxic effect reduction and to an increase in the active molecule affinity to the target site which is related to the increase in its lipophilic properties [1, 15].

As a part of cooperation with the group of Prof. Waldemar Priebe from the M.D. Anderson Cancer Center in Houston (USA), screening studies of a number of new anthracycline compounds in the aspect of their cytotoxic effect on the cells and intracellular transport in comparison with known and used anthracycline anti-

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Table 1. The effects of WP903 and ADR on induction of apoptosis in ME18 and ME18/R cells

ADR (μg/ml)	WP903 (μg/ml)	Living cells		Cells in early apoptosis		Cells in late apoptosis and necrosis	
		ME18	ME18/R	ME18	ME18/R	ME18	ME18/R
–	–	87.5 ± 1.5	73.2 ± 0.9	<1	<1	12.0 ± 1.0	25.3 ± 0.7
1	–	59.0 ± 7.9	65.0 ± 5.1	13.0 ± 3.5	<1**	28.7 ± 4.3	33.0 ± 6.0
5	–	33.7 ± 2.9	65.3 ± 2.2	$10.3 \pm 3.7*$	$2.7 \pm 1.2***$	56.3 ± 6.4	31.2 ± 1.5
–	0.2	42.7 ± 6.9	9.0 ± 1.2	22.7 ± 9.0	$59.7 \pm 3.8**$	34.3 ± 3.2	31.0 ± 2.3
–	2	<1	<1	$69.3 \pm 0.7*$	$71.0 \pm 2.1***$	30.0 ± 1.0	26.3 ± 1.5

24-h cell cultures were exposed to WP903 (0.2 and 2 μg/ml) or ADR (1 and 5 μg/ml) for the next 24 h. Cells kept in drug-free MEM were controls. After 24 h, the cells were treated with annexin V-FITC and PI (as described in Materials and methods). The results, presented as percent of cells ± SEM are the means of at least three independent experiments. *p=0.004, **p=0 .0042, ***p<0.0001

biotic, ADR, were performed at the Department of Biochemistry and Biopharmaceuticals of the NIPH. The studies have shown that the highest cytotoxic activity (*i.e.* relatively low values of IC_{50} and RF = resistance factor) can be attributed to the derivative known as WP903. It was also found that this compound enters the cells to the highest degree among the tested substances.

The purpose of this study was to evaluate the effect of a new synthesized anthracycline derivative WP903 on apoptosis induction in human melanoma cells, ME18, and an experimentally obtained ADR-resistant subline, ME18/R. The observed effect of apoptosis induction by WP903 was discussed in relation to the effects obtained for ADR.

This study was an attempt to determine the effect of the derivative on the induction of apoptotic processes depending on the concentrations used and the significance of apoptosis induction on the cytotoxic effect of anthracycline antibiotics.

Materials and methods

Reagents. ADR (adriamycin) was purchased from Fluka, Germany; antibiotics and antimycotics (penicillin 10 000 u/ml; streptomycin 10 mg/ml; amphotericin B 25 μg/ml) and PBS were obtained from IITD, Wrocław, Poland; apoptosis detection kit Annexin V came from Caltag Laboratories, USA; APO-BRDU[TM] kit for TUNEL assay was purchased from BD Pharmingen, USA; new synthesized anthracycline derivative WP903 was received from MD Anderson Cancer Center, Houston, USA.

Cell cultures. ME18-human melanoma cell line was obtained from the Centre of Oncology in Warsaw; ME18/R - human melanoma subline resistant to ADR was obtained experimentally in our laboratory (for this subline, resistance to WP903 was also shown).

Annexin assay by flow cytometry. 24-h cell cultures were exposed to ADR at concentrations of 1 and 5 μg/ml or to the WP903 derivative at concentrations of 0.2 and 2 μg/ml for subsequent 24 h. Afterwards, the cultures were rinsed twice using cold PBS, trypsinized and, after centrifugation, resuspended in a buffer (10 mM Hepes/NaOH, pH=7.4; 140 mM NaCl; 2.5 mM CaCl$_2$). The suspension density was approximately 10^6 cells/ml. Each cytometric tube was filled with 100 μl of the cell suspension; annexin V-FITC and PI were added to each tube according to Annexin V kit procedure. After gentle shaking

using "vortex", the tubes were incubated at room temperature in darkness for 15 min. The buffer (as above) was added and the samples were measured within 1 h using FACS Vantage flow cytometer of Becton Dickinson.

TUNEL assay by flow cytometry. Drug treatment and time exposure of cell cultures were as above. The cells, harvested in 1% (w/v) paraformaldehyde in PBS (pH=7.4), were fixed with use of 70% (v/v) ice cold ethanol according to the APO-BRDU[TM] kit procedure and stored at -20˚C until use. To follow TUNEL assay, the ethanol was removed by aspiration and the cells were washed twice with buffer containing 0.05% sodium azide. Then, the cell pellets were treated with DNA labeling solution (constisting of TdT enzyme and Br-dUTP). After 60 min incubation, the labeled cells were rinsed twice with buffer containing 0.05% sodium azide and resuspended in FITC-labeled anti-BrdU solution. Flow cytometry analysis of the cells (as above) was performed after 30 min storage in the dark at room temperature.

The concentrations of both compounds used in the experiment, *i.e.* 0.2 and 2 μg/ml of WP903 or 1 and 5 g/ml of ADR, represented values that were respectively lower or higher than the IC_{50} values determined for susceptible cells. These values were obtained in screening studies performed at our department [9]. The selection of concentrations was aimed at possible confirmation of greater efficacy of the derivative tested compared to ADR.

Statistical analysis. The statistical evaluation of the results was performed using Student's t-test, and C-Cochran and Cox test for unrelated samples.

Results

The results, presented in Table 1, were compared in the following system: lower and higher doses of WP903 (0.2 and 2 μg/ml) and lower and higher doses of ADR (1 and 5 μg/ml). As shown in Table 1, the WP903 derivative induced apoptotic events in human melanoma cells, regardless of the degree of sensibility to ADR and WP903, at concentrations several times lower compared to ADR concentrations.

In susceptible cells, ME18, the WP903 derivative used at a dose of 0.2 μg/ml had an apoptotic effect in more than 20% of cells, and at a dose of 2 μg/ml, approx. 70% of the cultured cells were in the early apoptosis phase (Fig. 1). Significant late apoptotic effects, observed in these experiments with use of annexin V-
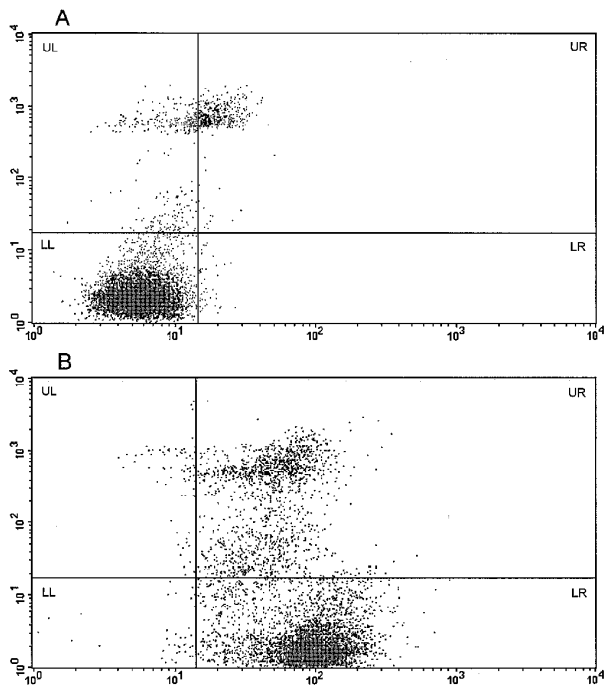
Fig. 1. Exemplary cytograms of ME18 cells stained with annexin V-FITC and PI; quadrants present as follows: LL-viable cells; LR-cells in early apoptosis; UR-cells in late apoptosis; UL-cells in necrosis. (**A**) untreated cells; (**B**) ME18 cells treated for 24 hrs with 2 μg/ml of WP903. X axis - fluorescence intensity of conjugates: cells-annexin V-FITC; Y axis - fluorescence intensity of conjugates: cells-PI.
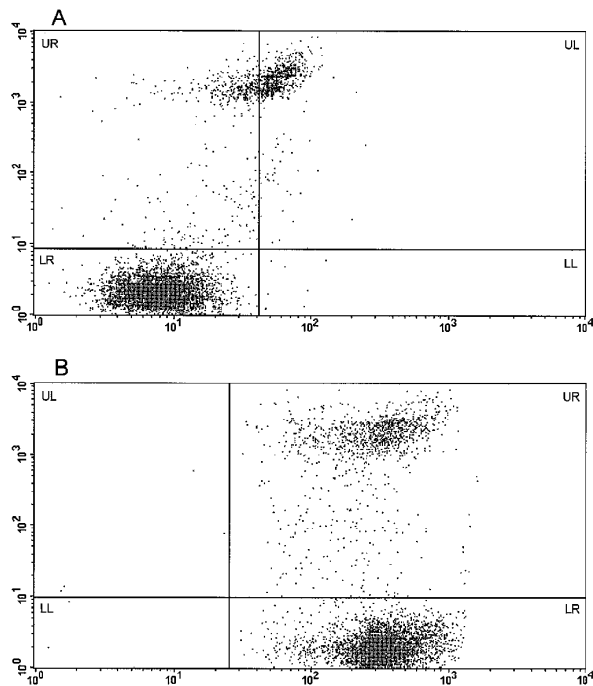
Fig. 2. Exemplary cytograms of ME18/R cells stained with annexin V-FITC and PI; quadrants present as follows: LL-viable cells; LR-cells in early apoptosis; UR-cells in late apoptosis; UL-cells in necrosis. (**A**) untreated cells; (**B**) ME18/R cells treated for 24 h with 2 μg/ml of WP903. X axis - fluorescence intensity of conjugates: cells-annexin V-FITC; Y axis - fluorescence intensity of conjugates: cells-PI.
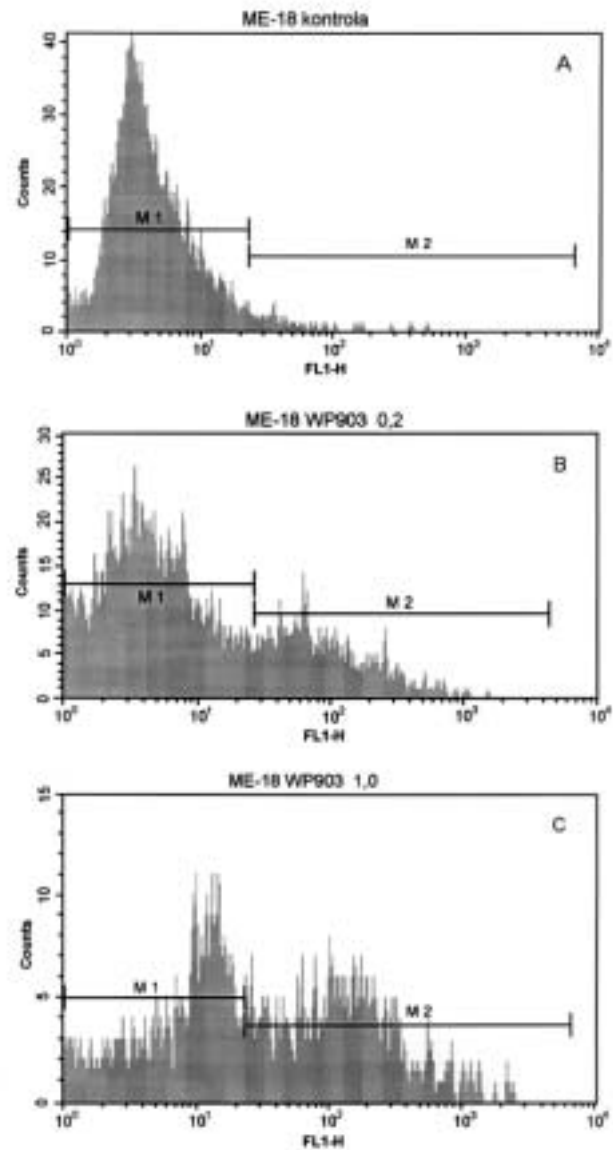
Fig. 3. Histograms of ME18 cells fixed and incubated with Br-dUTP in the presence of TdT enzyme. Br-dUTP was detected with a fluorescein labeled anti-BrdU mAb; the M1 and M2 gates demarcate non-apoptotic and late apoptotic populations, respectively. (**A**) untreated cells; (**B**) ME18 cells treated for 24 h with 0.2 μg/ml of WP903; (**C**) ME18 cells treated for 24 h with 2 μg/ml of WP903. X axis - fluorescence intensity of BrdU-fluorescein; Y axis - relative cell number.

FITC, were confirmed by TUNEL assay (Fig. 3). ADR at a dose of 5 μg/ml had a significantly lower apoptotic effect (approx. 10%) compared to the WP903 derivative; the dose of 1 μg/ml of ADR had no apoptotic effect at all. In the case of resistant cells, ME18/R, the WP903 compound caused early apoptotic changes in approx. 60% of cells even at a dose of 0.2 μg/ml. At the higher dose, 2 μg/ml, this effect was slightly increased (to approx. 70%, Fig. 2). In these cells, the early phase of

apoptosis was mainly observed. DNA fragmentation in the treated cells was observed at the level noted in the untreated cells. This observation was confirmed by TUNEL assay (data not shown).

Compared to ADR, WP903 showed significantly higher apoptosis induction ability in ME18/R cells, regardless of the dose used, although 2 μg/ml of WP903 was not efficient for ME18/R cells to induce DNA fragmentation.

Discussion

The techniques developed for identification of the apoptosis phenomenon in a given population of cells not only allow to determine the type of cell death but also the phase of biochemical events typical for apoptosis through the detection of characteristic features of each phase [5]. The apoptotic process detection using annexin is based on the phenomenon of membrane phosphatidylserine translocation onto the cell membrane surface as a result of early apoptotic changes. Annexin V is a Ca^{2+}-dependent protein, having high affinity for phosphatidylserine. The cells which stain only with annexin V-FITC are in the early phase of apoptosis. Positive staining with annexin V-FITC and PI (penetrating to dead cells) theoretically means late apoptosis; staining with PI only indicates cell necrosis.

The APO-BRDU^{TM} kit is a color staining method for labeling DNA breaks to detect late phase of apoptosis. The enzyme deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl (-OH) ends of double- and single-stranded DNA. After Br-dUTP incorporation, DNA break sites are identified by a FITC-labeled anti-BrdU monocloclal antibodies.

The results obtained in this study indicate that the WP903 derivative, contrary to ADR, has a very strong apoptotic effect in human melanoma cells (22% to 70% of cells) even when used at a low dose of 0.2 μg/ml. At the same time, this induction does not depend on the degree of cell susceptibility to this derivative. It shows that apoptosis is not a mechanism of anthracycline antibiotic action directly responsible for their cytotoxic activity.

This observation is consistent with the results obtained earlier in our laboratory for ADR [10], as well as partially consistent with the results obtained by other authors. According to Gewirtz [7], anthracycline antibiotics induce apoptosis only in low doses (up to 0.5 μg/ml), which was also shown by others [13] but this effect seems to have no significance for the cytotoxic activity of the drugs of this group.

In this study, the dose-dependent effect of WP903 on the occurrence of the apoptosis was especially observed in susceptible ME18 cells. In resistant cells, ME18/R, the concentration of 0.2 μg/ml was shown to be efficient to have maximum effect in this experiment. The result-

ing percentage of cells in early apoptosis (approximately 60%) changed very little at higher concentration of the compound. The lack of DNA fragmentation was noted after exposure of the cells to 2 μg/ml of WP903.

These observations are consistent with the report of Binaschi *et al.* [2] who found that there are so-called threshold concentrations, specific for each compound, that could trigger an early DNA degradation process. The level of this concentration probably depends on the degree of severity of DNA degradation that may occur during cell incubation with the tested compound, and the intensity of apoptotic effects depends on the type of apoptosis inductor and the cell exposure time [2, 12].

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