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NFκB ACTIVATION AND DRUG SENSITIVITY IN HUMAN NEOPLASTIC CELLS TREATED WITH ANTHRACYCLINES

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Abstract: NFκB (nuclear factor κB) is a transcription factor controlling, among others, cell proliferation and apoptosis. The potent activators of NFκB are anthracyclines which can activate apoptotic processes. As shown by some authors, NFκB activated by these drugs well correlated with their cytotoxic activity. The aim of this study was to assess the effects of doxorubicin (DOX) and its analogs (annamycin, WP903) on the NFκB activity in human melanoma cells: a sensitive (ME18) and a resistant to DOX (ME18/R) and its possible correlation with cell sensitivity to these drugs. In the studies, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, ELISA test and confocal microscopy were used. As was shown, DOX, 1.7; 8.6 μM, strongly induced NFκB in ME18 cells. Annamycin (ANN), 0.3; 3.0 μM and WP903, 3.0 μM induced NFκB in ME18/R cells. PDTC (pyrrolidine dithiocarbamate) – NFκB inhibitor made ME18/R cells more sensitive to ANN and WP903 but did not affect cytotoxicity of DOX in ME18 cells. These results suggest that the influence of NFκB activation on cytotoxicity of anthracyclines is highly drug- and cell-specific.

Keywords: anthracyclines; NFκB; cytotoxicity

NFκB is a transcription factor which was discovered by Ranjan Sen and David Baltimore in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin (1). Activated NFκB regulates expression of over 200 genes that control the immune system, inflammation and growth, among of them, these are genes involved in both, the induction and suppression of apoptosis (2-5).

NFκB is present in all cells in a resting state in the cytoplasm. It consists of a family of Rel-domain-containing proteins as Rel A (also called p65), Rel B, c-Rel or p50. Similarly, a family of anchorin-domain-containing proteins have been identified that keep NFκB in its inactive state within the cytoplasm. These include IκBα, IκBβ or IκBγ (3, 4).

Following different stimuli, IκBα is phosphorylated by the IKK (IκBα kinase) kinase complex, polyubiquitinated and degraded. That process leads to the release of the p50, p65 which then translocate to the nucleus and binds its specific 10-base-pair *consensus site* (3).

Number of studies *in vitro* and *in vivo* prove participation of NFκB in cytotoxic activity of anti-cancer drugs. As was shown by Tabata et al. (6), treatment of human nonsmall cell lung carcinoma cells (NSCLC-3 or NSCLC-5) with the topoisomerase I poison SN-38 or the topoisomerase II poison etoposide (VP-16) led to activation of NFκB before induction of apoptosis. Mitoxantrone activated NFκB and stimulated IκBα degradation in the promyelocytic leukemia cell line HL60 but not in the resistant HL60/MX2 cells (7). The proapoptotic role of that transcription factor was noted also by Mabuchi et al. (8) in human ovarian papillary adenocarcinoma cell line Caov-3 treated with paclitaxel in *in vitro* and *in vivo* studies. Paclitaxel transiently activated the cascade of protein phosphorylation which led to a transient increase in NFκB activity. Treatment with IκBα phosphorylation inhibitor – BAY 11-7085 decreased the viability of cells treated with paclitaxel. Moreover, treatment with BAY 11-7085 increased the efficacy of paclitaxel-induced inhibition of intraabdominal dissemination and production of ascites in athymic nude mice inoculated intraperitoneally with Caov-3 cells.

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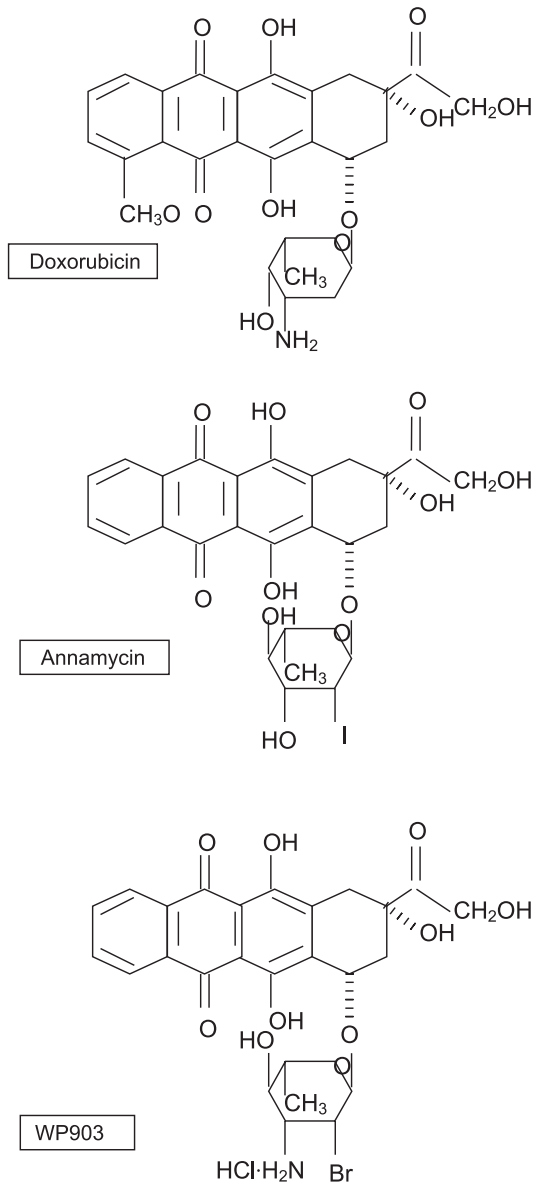
The potent activators of NFκB are also anthracycline anticancer drugs (9, 10). Similar to other NFκB activators, their effects appear to involve reactive oxygen species but also DNA lesions generation. It has been shown that anthracyclines activate the events of programmed cell death (apoptosis) in cells which may be a mechanism underlying their therapeutic efficacy in certain tumor types. Above studies could point to the pro-apoptotic role of NFκB. Recent studies have shown that activation of this transcription factor induced by anthracycline analogs well correlated with cytotoxic activity of these compounds (10).

The search of the main cytotoxic mechanisms of anthracyclines is the pivotal matter because of the drug resistance which is the big obstacle in antitumor therapy. So, the role of NFκB may be the chance to find some explanation of these problems.

The aim of this work was to evaluate the influence of DOX and its analogs: ANN and WP903 (Scheme 1) on the activity of NFκB in human melanoma cells: a sensitive – ME18 and a resistant to DOX – ME18/R and to study the correlation between these events and cytotoxic activity of the tested drugs.

Cytotoxic activity of anthracyclines were studied with MTT assay. Activation of nuclear transcription factor was determined with ELISA test and confocal microscopy.



Scheme 1.

MATERIALS AND METHODS

Drugs

DOX was purchased from Fluka, Germany; WP903 and ANN were synthesized by Prof. Waldemar Priebe, The University of Texas, M.D. Anderson Cancer Center.

Cell cultures

ME18 – human melanoma cell line was a gift from M. Skłodowska–Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; ME18/R – the subline resistant to DOX was obtained in our laboratory (11).

Cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and antibiotics. ME18/R cells were maintained in MEM with DOX, 0.04 μM. All cell cultures were mycoplasma-free.

MTT test

The suspensions of cells were diluted in MEM supplemented as given above to 10^5 cells/mL. 0.05 mL of each suspension was placed into individual wells on a 96-well multiplate. Then, 0.05 mL of each drug solution in MEM was added for 48 h. The MTT test was proceeded as described earlier (12). The final concentrations of DOX, ANN and WP903 ranged from 0.09 to 10.0 μM. To confirm the role of NFκB in cytotoxicity of the tested drugs, the studies with the universal inhibitor of NFκB – PDTC were performed (13). The IC_{50} values (the drug concentration which inhibits the growth of the cell culture at 50%) for DOX, ANN and WP903 were determined in the cell cultures preincubated with PDTC, 0.2 μg/mL for 24 h (that concentration was chosen

experimentally as the highest and non cytotoxic) and then, exposed to the drugs ranged from 0.09 to 10.0 μM for 24 h.

The doses of anthracyclines used in further experiments were defined on the basis of our earlier studies (14, 15).

Nuclear extracts preparation

The 24-h cell cultures were exposed for 24 h to the following concentrations of the drugs tested: DOX – 1.7; 8.6 μM; ANN and WP903 – 0.3; 3.0 μM. After that time, the cells were harvested with PBS (phosphate buffered saline) into the Eppendorf tubes and centrifuged. The nuclear extracts were prepared strictly according to the procedure given earlier (16).

Confocal microscopy

The cover glasses with the confluent cell cultures on the surface were fixed for 5 min in acetone (-20^{o}C), washed with PBS and then, blocked with normal goat serum (Oncogene NSO2L) for 30 min at room temperature. After washing with PBS, the cells were overlaid for 1 h with polyclonal antibodies (1 : 66): NFκB (p65RelA) (ab-1) (Oncogene PC137) or NFκB (p50) (ab-1) (Oncogene PC136), previously complexed with the fluorochromes Alex 647 (Z-25302 Molecular Probes) or Alex 488 (Z-25302 Molecular Probes), respectively. Next, the complexes antibody-fluorochrome were fixed with paraformaldehyde, 4% for 15 min and then washed with PBS. After dying with propidium iodide (PI) and RNase, the glasses were placed in the mounting medium – Prolong™ Antifade kit (Molecular Probes) and analyzed with use of confocal microscopy Olympus FV500 with the lasers: Ar – 488 nm; He-Ne – 543 nm and He-Ne – 633 nm. Microscopy images were analyzed with use of program FluoView, ver.4.3.

The increase in fluorescence intensity of the complexes antibody–fluorochrome measured throughout the nuclei of the individual cells reflected the translocation of p50 or p65 from cytoplasm to the nuclei being a consequence of NFκB activation.

ELISA test

BD™ Transfactor Kit provides rapid detection of specific transcription factor activity in cell extracts. Using an enzyme-linked immunosorbent assay – based format, the Transfactor kit detects DNA binding by NFκB. Each kit contains the plate with the consensus binding sequence for NFκB, p50 or p65 subunits, coated in wells. Nuclear cell extracts were incubated in the wells to allow NFκB subunits bind to their consensus sequences. Bound subunits were detected with specific primary antibodies, i.e. p50 with whole rabbit antiserum; p65 with rabbit polyclonal IgG. A horseradish peroxidase – conjugated secondary antibody (goat anti-rabbit IgG-HRP) was then used to detect the primary antibody. The enzymatic product formed in colorimetric reaction could be measured with an absorbance microtiter plate reader at 650 nm. Positive control was the extract from HeLa cells activated with TNFα.

Statistical analysis

The statistical evaluation of the results obtained in this work was performed using Student's t – test and Cochrane's Cox test for unrelated samples.

RESULTS

Cytotoxic activity of anthracyclines

As shown in Table 1, parental ME18 cells exhibited much more sensitivity to all anthracyclines tested than the ME18/R cells ($p < 0.05$ for all results). ANN and WP903 occurred more effective than DOX for ME18 cells. WP903 was shown as the most active towards ME18/R cells.

NFκB activation

As shown in Table 2, the treatment of ME18 cells with all tested anthracyclines: DOX, ANN and WP903 caused activation of NFκB. DOX at both concentration induced translocation of p50 noted as the about nine-fold increase in fluorescence signal as referred to the untreated cells and caused the dose dependent effect of p65 translocation, i.e. 25- and above 80-fold increase in fluorescence signal under treatment with 1.7 or 8.6 μM of DOX, respectively. These effects were the strongest among all measured in these studies. The weaker effects, inversely correlated with the doses used and associated only with p65 subunit were observed in these cells exposed to ANN. Six- and two-fold increase in fluorescence signal was noted at 0.3 and 3.0 μM of ANN, respectively.

The weak (about two-fold) increase was observed in the case of p50 translocation under treatment with WP903, 3.0 μM. In fact, no activation was noted in p65 translocation after exposure of these cells to 0.3 μM and 3.0 μM of that compound.

In the case of ME18/R cells (Table 2), DOX did not activate NFκB. The activation was observed only as the translocation of p50 subunit after treatment with ANN at both concentrations and with

WP903 at the higher concentration, 3.0 μM (about 5-fold increase in fluorescence signal in all three cases). It is worth of noting that these results were confirmed using an enzyme-linked immunosorbent assay (ELISA test) (data not shown).

Table 1. IC_{50} values [μM] determined with use of MTT test.

	CELLS	
DRUG	ME18	ME18/R
DOX	12.21±3.18	48.68±2.06
ANN	0.90±0.17	44.15±2.26
WP903	1.48±0.41	5.41±0.43

The results represent the mean ± SEM (n = 8-16). The cells were treated for 48 h with drugs ranged from 0. 09 – 10.0 μM. Then, the MTT test was performed as was described in Materials and Methods. All IC_{50} values determined for ME18 cells were statistically different from that obtained for ME18/R cells, respectively ($p < 0.05$ for all results).

DISCUSSION AND CONCLUSIONS

As was shown in this work, anthracyclines are the activators of NFκB in human neoplastic cells which is consistent with the data obtained by Ashikawa et al. (10) within studies on human myeloid KBM-5 cells. The current experiments did not confirm that DOX is the weaker inducer of NFκB than the new anthracyclines. Ashikawa et al. (10) showed that DOX induced the maximal NFκB activation in KBM-5 and H1299 cells at about 50-fold higher concentration than the new anthracycline WP744, although at the same dose as other analog WP631. The effective doses of DOX and WP631 were about 50-fold higher than IC_{50} values. In our work it was shown that DOX activated NFκB in ME18 cells to much stronger extent than ANN or WP903, respectively, and all drug doses tested in the studies were not such drastically different from each other as was described by Ashikawa (10). The corre-

Table 2. Effects of DOX, ANN and WP903 on NFκB activation in ME18 and ME18/R cells.

Cells / Drug [μM]	ME 18		ME 18/R	
	p 50	p 65	p 50	p 65
–	200	48	80	285
DOX 1.7	1650	1180	120	172
DOX 8.6	1800	>4000	112	138
ANN 0.3	305	305	365	220
ANN 3.0	160	90	455	165
WP 903 0.3	140	80	112	86
WP 903 3.0	380	75	470	370

Fluorescence intensity values originated from complexes: antibodies NFκB (p65RelA) (ab-1) or NFκB (p50) (ab-1) with fluorochrome in nuclei analyzed with program FluoView, ver.4.3 in the confocal microscope.
The 24 – h cell cultures were treated for 24 h with the drugs as follows: DOX – 1.7; 8.6 μM; ANN and WP903 – 0.3; 3.0 μM. Then the cells were prepared for analysis as was given in Materials and Methods.

NFκB activity and cytotoxicity of anthracyclines

As was shown in Tables 1 and 2, the correlation between increased sensitivity to DOX and NFκB activation was noted in ME18 cells. In turn, the activation of that transcription factor by ANN and WP903 was correlated with diminished sensitivity to these drugs in ME18/R cells but seems to be not important for sensitivity of ME18 cells.

As shown in Table 3, inhibition of NFκB by PDTC made ME18/R cells more sensitive to ANN and WP903. That inhibitor did not affect cytotoxicity of DOX towards ME18 cells (data not shown).

Table 3. Influence of NFκB inhibition by PDTC on cytotoxicity [IC_{50}] of ANN and WP903 in ME18/R cells.

DRUG	IC_{50} [μM]
ANN	44.15 ± 2.26
ANN +PDTC	3.21 ± 0.45
WP903	5.41 ± 0.43
WP903 + PDTC	1.05 ± 0.30

The cells were preincubated with PDTC, 0.2 μg/mL for 24 h and next, exposed to ANN or WP903 ranged from 0.09 – 10.0 μM. After 48 h the MTT test was performed as was described in Materials and Methods.
All differences noted in IC_{50} values for each drug were statistically significant ($p < 0.05$).

lation observed between IC_{50} values and NFκB activity in ME18 cells compared with ME18/R cells treated with DOX (Tables 1 and 2) was not confirmed in the study with use of NFκB inhibitor – PDTC. These results are not in agreement with Bian et al. (17) who noted that in N-type neuroblastoma cells, NFκB activation mediated cell killing by DOX.

The effects of anthracyclines on NFκB are cell specific. Our earlier studies with use of human cervix carcinoma cells HeLa and KB-V1, subline resistant to vinblastine shown almost identical cytotoxicity of DOX, ANN and WP903 towards both cell lines which was independent on the NFκB activation profiles (data not shown). According to Wu and Kral (4), the specificity of NFκB may reside in the cell type targeted; not all cell types respond equally to a given stimulus, either because they lack the cognate receptor or the required signal transduction molecules. Also, NFκB may not be sufficient for full transcription, as other transcription factors are also required or because of different availability of NFκB hetero- and homodimers. The results obtained in ME18/R cells exposed to ANN or WP903 are in agreement with Ma et al. (18) who showed that the increased NFκB activity is associated with increased tumor cells survival in multiple myeloma.

It is worth of noting that the effects of ANN and WP903 on NFκB were studied not only in human melanoma cells ME18 but also in original subline ME18/R, resistant to DOX, methotrexate and vinblastine obtained in our laboratory and that the studies on the role of NFκB in cytotoxicity of ANN (being currently evaluated in clinics) and novel analog WP903 are pioneering.

On the basis of the results described above it is possible to conclude that:

– anthracyclines are the activators of nuclear transcription factor – NFκB;
– the influence of NFκB activation on cytotoxicity of anthracyclines seems to be highly drug- and cell-specific;
– the changes in molecular structure of the drug may imply the diversity in biological response.

Whether NFκB activation is beneficial or harmful for cancer is still controversial. The development of novel therapeutics targeting NFκB requires full understanding of its role in pathology and physiology. These preliminary studies suggest that NFκB inhibition may, at least partly, overcome chemoresistance and may allow the doses of chemotherapeutic agents to be reduced with antitumor effects without significant toxicity.

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