Eucaryotic DNA topo I and II are nuclear enzymes that function in almost all aspects of DNA metabolism including replication, transcription, recombination, repair and chromosomal condensation, by controlling the topological state of DNA (1–3). Topo II is an ATP dependent enzyme that catalyzes DNA strand passage, the pivotal process in replication, transcription, recombination etc. As part of this breakage and religation process the intermediate generated is a cleavable complex between DNA and topoisoasemerase II. This complex is the target for topoisoasemerase II inhibitors like epipodophyllotoxins, actinomycin D or anthracyclines. Stabilization of cleavable complexes by the topoisoasemerase II inhibitors leading to DNA lesions and next to apoptosis is the most common mechanism of drug resistance reflected in reduced formation of the complexes due to decreased amounts and/or activity of topoisoasemerase II. The aim of this study was to characterize human melanoma and human cervix carcinoma cells differing in sensitivity to doxorubicin and anthracycline analogs, annamycin and WP903 for topoisoasemerase II protein and gene expression with use of Western blot and RT-PCR. As shown, no significant differences in topoisoasemerase II protein level were noted between the cell lines tested. These results were confirmed at the gene expression level. The current study points to the fact that topoisoasemerase II protein or gene expression are not the reliable marker of cell sensitivity to anthracyclines but these observations do not exclude the potential mutations in topoisoasemerase II gene or some posttranslational changes in that protein which requires further studies.

**Keywords:** topoisoasemerase II; anthracyclines; neoplastic cells; RT-PCR

Many topo II interacting drugs are DNA intercalators such as anthracyclines, actinomycins, ellipticines, alkaloids and few are non-intercalators such as etoposide and teniposide (3, 6).

In tumor cells selected for resistance to topo II–targeted drugs, the most common mechanism of drug resistance involves reduced formation of cleavable complexes due to the expression of decreased amounts and/or activity of topo IIα (3, 4). Some authors point to the role of topo IIα – one of the isoform of topo II in multidrug resistance and, in this way, topo II condition in the cells may be the major factor in sensitivity to topo II inhibitors (7, 8).

The anthracyclines are some of the most valuable anticancer drugs in clinical use. Among these DOX (Scheme I) is the best known, being the integral part of the treatment of malignancies such as breast lung, ovary carcinoma or soft tissue sarcomas and leukemias (3, 8). It is generally accepted, however, that cellular drug resistance is one of the major reasons for failure in treatment. Because of that, the relative importance of drug interference in topo function has been emphasized in
several studies on tumor cells resistant to DOX (3, 7). It seemed to be interesting to study the condition of topo II in the cell lines with varying levels of resistance to anthracyclines.

The aim of this study was to characterize some neoplastic cells, more or less sensitive to DOX and anthracycline analogs, ANN and WP903 (Scheme 1) for topo IIα gene expression and protein levels to indicate the possible role of that isoform in anthracycline resistance phenomenon. For that purpose, RT-PCR and Western blot methods were used.

EXPERIMENTAL

Materials

Drugs. DOX was purchased from Fluka, Germany; ANN and WP903 were received from MD Anderson Cancer Center, Houston TX, USA.

Reagents. MEM (Minimum Eagle’s Medium), Gibco; PBS (phosphate buffered saline), Institute of Immunology and Experimental Therapy, Wroclaw; Hepes (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid]; DTT (dithiotreitol); PMSF (phenylmethylsulfonyl fluoride) and Antibiotic antimycotic (penicillin 10000 u, streptomycin 10 mg, amphotericin B 25 µg/mL) were purchased from Sigma.

Cells. ME18 – human melanoma cells were obtained from M. Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland; ME18/R- it’s subline resistant to DOX was obtained experimentally (9); HeLa – human cervix carcinoma cells; KB -V1- its subline resistant to vinblastine were purchased from German Collection.

Cells were grown in MEM supplemented with 10% fetal calf serum and antibiotics. ME18/R cells were maintained in MEM with DOX, 0.02 µg/mL and KB-V1 cells in MEM with vinblastine, 0.5 µg/mL, given once a month.

Methods

MTT assay. The suspensions of the cells were diluted in MEM to 10^5 cells/mL and 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 at double strength dilution for 48 h. The final concentrations of DOX, ANN and WP903 ranged from 0.058 to 5.8 µg/mL. The cells treated as the controls were kept in drug-free MEM. MTT test was performed as reported earlier (10). The IC₅₀ values were determined as the concentrations of the drugs required for 50% cell growth inhibition.

Preparation of nuclear extracts. The cell pellets were washed with cold PBS and resuspended in lysis buffer A (1 M Hepes, pH = 7.9/2 M KCl/0.5 M EDTA, pH = 8.0/0.1 M EGTA, pH = 7.0) with addition of 0.1 M DTT, 100 mM PMSF, leupeptin 1 mg/mL, aprotinin 1 mg/mL and benzamidine 250 mg. During one hour incubation on ice with intermittent vortexing, the cells were disrupted and nuclei were released visible as the transparent pellet. After one hour, non-ionic detergent was added to all samples. The supernatants (cytoplasmic extracts) were collected with prior vortexing and spinning. Nuclear extracts were obtained by incubation of the remaining pellet on ice for 2 h in lysis buffer B (1 M Hepes, pH = 7.9/5 M NaCl/0.5 M EDTA, pH = 8.0/0.1 M EGTA, pH = 7.0) with addition of 0.1 M DTT, 100 mM PMSF, leupeptin 1 mg/mL, aprotinin 1 mg/mL, benzamidine 250 mg and with frequent intermittent vortexing. The extracts were kept at −70°C for further analysis.
Western blot. The extracts were resolved by 12% SDS PAGE electrophoresis (each well was loaded with 50 µg of protein) and electroblotted onto a nitrocellulose membrane, 0.45 nm. After blocking with 3% unfat milk, topo IIα protein was detected with rabbit polyclonal antibody, 200 µg/mL IgG (1:100), Santa Cruz Biotechnology Inc., USA (corresponding to fragment of the carboxy terminus of DNA topo IIα of human origin). Secondary antibody was polyclonal goat anti-rabbit IgG-HRP conjugated (1:500), Dako, Denmark. Proteins were visualized with HRP Color Development Reagent (4CN(4-chloro-naphthol)).

RT-PCR. Total RNA of the tested cells was isolated with use of the NucleoSpin®RNA II kit (Macherey-Nagel). Synthesis of cDNA at the RNA template and amplification of that reaction products was realized with the QIAGEN®OneStep RT-PCR Kit Handbook (Qiagen). The following primers were designed: 5’-ACG ATG TCG CAG AAG AGA GG-3’; the expected product size was ca. 1110 bp. The transcription region defined by the primers used was between 631-1740 bp. The PCR products were analyzed by electrophoresis on 1.5% agarose gel with ethidium bromide (0.1 µg/mL) in TAE buffer 1x, and next, verified by comparison with MWS 50-3000 bp, DirectLoad™ Step Ladder (Sigma).

Statistical analysis. The statistical evaluation of the results was performed using Student’s t-test and Cochrane’s Cox test for unrelated samples.

RESULTS

Cytotoxic activity

Cytotoxic activity of the tested drugs was determined with use of MTT test. As shown in Table 1, parental ME18 cells exhibited much more sensitivity to all anthracyclines tested than the subline ME18/R (p<0.05 for all results). The RF-s values for DOX and WP903 were at the same level. The highest RF was observed in the case of ANN. HeLa and KB-V1 cells exhibited comparable sensitivity to all drugs tested.

Topo IIα expression

As was revealed in Western blot analysis (data not shown), topo IIα was present in nuclear extracts in all cell lines tested, but no significant differences in topo IIα bands intensity were noted between them. It means that in all cases topo IIα protein found in nuclear extracts was detected at the similar levels. To confirm these results RT-PCR analysis was done.

As shown in Figure 1, the expected products in size ca. 1000 bp which directly corresponded to topo IIα cDNA and indirectly to topo IIα mRNA, have occurred in all cell lines tested. The semiquantitative study on the band intensity revealed no difference in the level of topo IIα cDNA which would have been correlated with cell sensitivity to anthracyclines.
DISCUSSION

Among the opinions that topo IIα has the pivotal role in cytotoxicity of anthracyclines and that the topo IIα protein expression can be predictive marker of drug resistance (7, 8, 11-13), there are the data consistent with our observations. According to Turley et al. (4), the expression of topo II isoforms may be responsible for the drug response of certain tumor types but the lack of response to chemotherapy in tumors treated with topo II targeting drugs is not caused by a lack of the target enzyme.

Satherley et al. (14) studied the expression of topo II isoforms in 29 choroidal melanomas for which chemosensitivity assay data for DOX were also available. As they noted, the level of topo IIα did not reliably predict sensitivity or resistance of the tumor tested. Vassal et al. (15) did not find any relationship between drug sensitivity to DOX and expression of topo IIα in athymic mice bearing subcutaneous medulloblastoma and glioblastoma xenografts. According to Den Boer et al. (16) topo IIα may be an indicator of the proliferative status of the cells without a direct relationship with anthracyclines resistance in acute leukemia.

As was observed by Son et al. (3), a quantitative reduction in topo IIα may contribute to the resistance of MKN cells (human stomach adenocarcinoma cell line) to DOX and other topo II targeted drugs. Following these authors, the current study may point to the fact that topo IIα protein expression is not the reliable marker of cell sensitivity to anthracyclines but, on the other hand, the level of protein expression noted with use of Western blot and RT-PCR can not exclude the potential mutations in topo IIα gene or some posttranslational changes in topo IIα protein.

Such events may include, for example, amplification or deletion in topo IIα gene or modifications in phosphorylation of topo IIα catalytic domain (5, 17).

Following Nielsen et al. (18) alteration in cell sensitivity to topo II targeted drugs may result from quantitative changes e.g., decreased levels of topo II protein or qualitative changes, e.g. mutations or altered enzymatic function by posttranslational modification of the phosphorylation status of the protein.

These data help to create some ideas for our further work. The genetic regularity of topo IIα protein in the cell tested should be checked: and also it is needed to confirm that topo II poisoning which results in stabilization of cleavable complexes is the mechanism differentiating anthracycline-sensitive and -resistant cells.

REFERENCES


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