Topoisomerase IIα expression in acute myeloid leukaemia cells that survive after exposure to daunorubicin or ara-C

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Received June 30, 2009; Accepted August 5, 2009

DOI: 10.3892/or_00000597

Abstract. Patients diagnosed with acute myeloid leukaemia are often treated with a combination of daunorubicin and 1-ß-D-arabinofuranosylcytosine (ara-C). Both daunorubicin and ara-C exert their effects in the cell nucleus but by different mechanisms, i.e. daunorubicin causes double stranded DNA breaks by inhibition of the nuclear enzyme, topoisomerase (topo) IIα, whereas ara-C is an anti-metabolite that integrates with DNA during DNA synthesis and causes cell cycle arrest. Despite the initial efficacy of these drugs, resistance often develops in the clinical setting. The mechanisms underlying clinical resistance to these drugs are poorly understood, but may be associated with an increase in the proportion of topo IIα negative cells. Therefore, the aim of this study was to determine whether daunorubicin treatment results in increased numbers of topo IIα negative subpopulations *in vitro*. Acute myeloid leukaemia cells isolated from 12 consenting patients were treated for 24 h with increasing concentrations of daunorubicin or ara-C and the proportion of topo IIα-negative cells in surviving cell populations determined by flow cytometry. Treatment with daunorubicin, but not ara-C, resulted in a significant increase in the proportion of topo IIα negative cells (p=0.0023). These results suggest that daunorubicin may act by cell cycle arrest and/or by selection of pre-existing topo IIα negative subpopulations. Both of these mechanisms can theoretically contribute to a reduced efficacy of a second dose of daunorubicin. The clinical relevance of these interactions should be further elucidated in experimental and clinical studies.

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Key words: acute myeloid leukaemia, topoisomerase, daunorubicin, 1-ß-D-arabinofuranosylcytosine, flow cytometry

Introduction

A combination of daunorubicin and 1-ß-D-arabinofuranosylcytosine (ara-C) is the standard induction treatment for acute myeloid leukaemia (AML) (1). Daunorubicin exerts its effect in the cell nucleus by interaction with DNA and the enzyme, topoisomerase (topo) IIα. Ara-C is a nucleoside analogue that requires phosphorylation to ara-CTP to become an active cytotoxic drug (2). Similarly to daunorubicin, ara-C exerts its effect in the cell nucleus and causes DNA damage by incorporation into the DNA molecule (3). Unfortunately, only 50-60% of patients treated with cytostatic drugs such as daunorubicin and ara-C will enter complete remission, and the long-term survival rate is only 10-20% (1). A major reason for this poor outcome is believed to be a result of intrinsic or acquired resistance to cytostatic drugs.

Several cellular mechanisms have been identified that are involved in drug resistance. One mechanism that may play a key role in resistance to daunorubicin is expression of the target molecule, topo IIα. Topo IIα changes the tertiary structure of DNA and plays an essential role during transcription and duplication of DNA (4). To act as a functional enzyme it requires dimerisation, mainly in the form of homodimerisation. The topo IIα dimer causes DNA double-strand breaks that are fixed by disulphide bonds, the so-called cleavable complex. Under ATP hydrolysis the DNA break is religated and the dimer dissociates from the DNA. Daunorubicin stabilises the cleavable complex, which results in permanent DNA double strand breaks and thereby induces cell death. Ara-C is a topo IIα independent drug, but causes DNA damage that triggers DNA cleavage by topo IIα (5).

During cell proliferation in non-malignant cells the expression of topo IIα increases during the S, G2 and M phases and rapidly decreases after mitosis (6). Several studies have reported that topo IIα expression in malignant cells is less cell cycle-dependent than in non-malignant cells and that topo IIα can also be significantly expressed in the G0/G1 phase (7-9). The expression of topo IIα during G0/G1 may explain the treatment efficacy of topo IIα inhibitors in low proliferating malignant diseases such as AML. However, studies have not been able to correlate the topo IIα expression to clinical outcome (10-13).

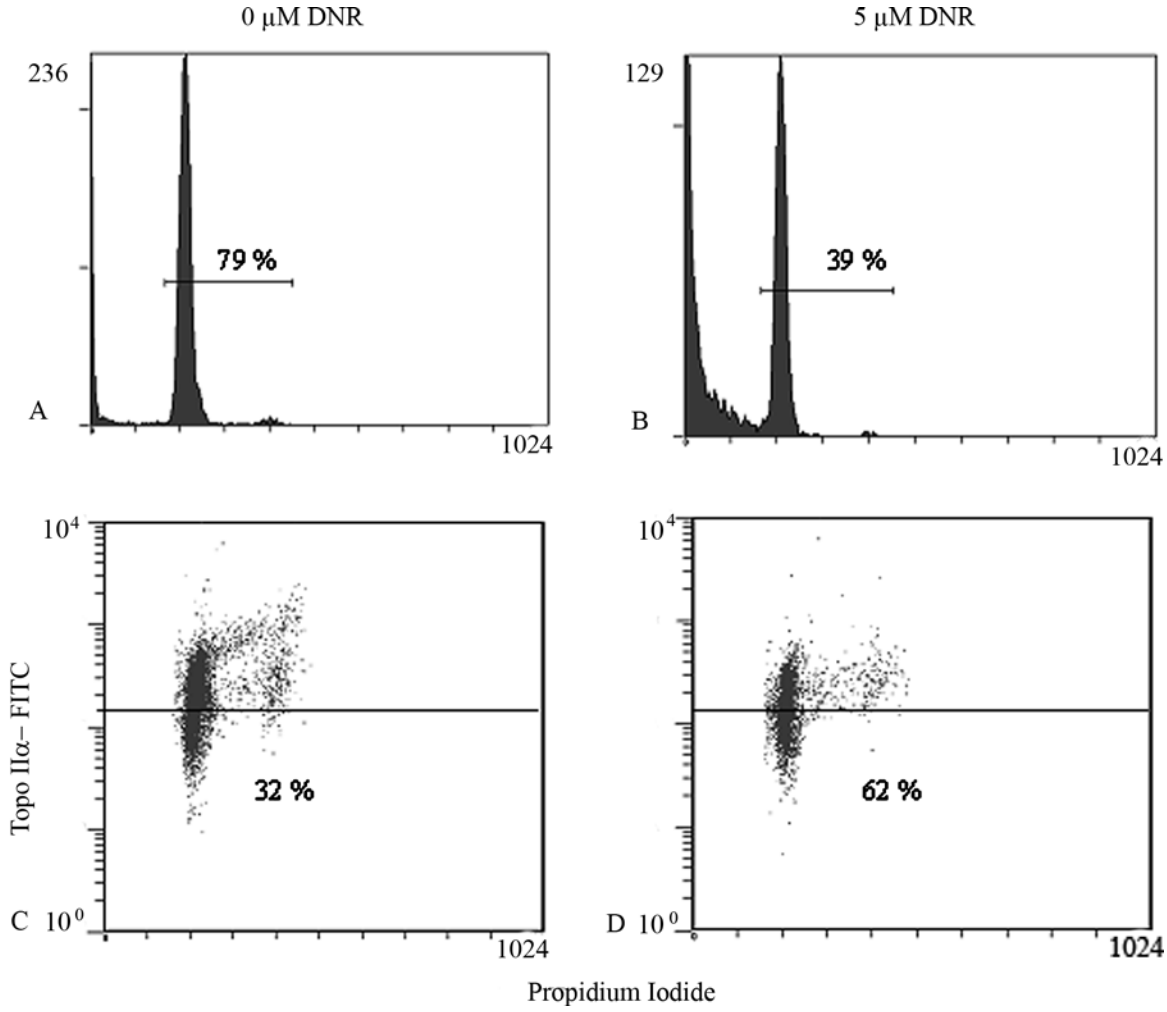

Figure 1. Topo IIα expression by human leukaemia cells after 24 h of incubation with 0 and 5 μm daunorubicin (DNR). (A and B) Gating was set on the PI-histogram to exclude sub G0/G1 events. (C and D) Cells included in the gate were analysed for topo IIα expression. Cells below the cut-off line in the dot plot were assessed to be topo IIα negative cells.

In this study, we incubated human leukaemia cells with increasing concentrations of daunorubicin or ara-C and analysed the expression of topo IIα in the surviving cell population. The aim of the study was to investigate the presence of topo IIα negative subpopulations that theoretically could result in clinical drug resistance.

Materials and methods

Patients. At diagnosis, blood or bone marrow samples from 12 adult AML patients were obtained with the approval of the ethics committee at Örebro University Hospital and informed patient consent. Mononuclear cells were isolated with Histopaque -1077 (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

Culture of cells with cytostatic drugs. Cells $1x10^5$/ml (derived from 12 patients, as described above) were cultured in 25 cm^2 cell culture flasks in a final volume of 10 ml. During incubation, cells were cultured in medium consisting of 87% RPMI-1640 (Gibco, Paisley Scotland, UK), 2.5% 1 M HEPES (Gibco) and 10% foetal bovine serum (Gibco). Daunorubicin (Aventis Pharma, Bromma, Sweden) and ara-C (Pfizer AB, Sollentuna, Sweden) were used at final concentrations of 0.1, 0.5, 2 and 5 μM (9 ml medium + 1 ml cytostatic drug diluted in RPMI-1640). Due to a limited number of cells, in three cases the cells were incubated with ara-C only at the highest concentration. As a drug-free control, cells were incubated in medium only. Incubation with daunorubicin was terminated after 1 h by centrifugation at 300 x g for 5 min and re-diluting in 10 ml fresh medium. Drug-free controls and ara-C were incubated continuously. All samples were cultured for 24 h in a humidified incubator (37˚C, 5% CO_2) and were performed in triplicate; two flasks were used for topo IIα analysis and one flask was used for determination of viable cells.

Topoisomerase IIα assay. Analysis of topo IIα expression was performed before and after cell culturing. All incubation steps were performed on ice in two parallel tubes. The mononuclear cells were lysed with 0.1% Igepal CA-630 in citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 5% dimethylsuphoxide in distilled water pH 7.6) for 10 min. Isolated nuclei were then washed once with ice-cold Dulbeccos PBS (Gibco) and pelleted by centrifugation at 500 x g for 5 min

Table I. Cell viability, before and after incubation of human leukaemia cells with a range of concentrations (Conc.) of daunorubicin (DNR) or ara-C, presented as mean values (range).

	% Viable cells	
Conc.	DNR	ara-C
0 μM, 0 h	97 (92-99)	97 (92-99)
0 μM, 24 h	76 (58-90)	76 (58-90)
0.1 μM, 24 h	66 (43-91)	74 (48-93)
0.5 μM, 24 h	60 (33-90)	74 (38-92)
2 μM, 24 h	44 (15-75)	73 (51-90)
5 μM, 24 h	31 (10-56)	69 (49-89)

at 4˚C. Anti-topo IIα [clone Ki-S1 (Chemicon, Temecula, CA, USA)] diluted 1:20 in citrate buffer was added to one tube and to the other tube IgG2a (Dako, Glostrup, Denmark) diluted 1:10 in citrate buffer was added. Both tubes were incubated for 15 min. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse diluted 1:10 in citrate buffer was used as a secondary antibody, and incubated for 15 min. For DNA-staining, propidium iodide (PI) (100 μg/ml in citrate buffer) was added and incubated for at least 15 min before flow cytometry analysis.

Determination of viable cells. 7-Amino-actinomycin D (7-AAD) (BD Biosciences Pharmingen, San Jose CA, USA) was used to determine the proportion of viable cells before and after cell culture by exclusion of non-viable cells. Cells were incubated with 0.25 μg 7-AAD for 10 min at room temperature and protected from light, then diluted with PBS and analysed by flow cytometry.

Flow cytometry analysis. Flow cytometry analysis was performed using an EPICS® ALTRA™ (Beckman Coulter, Fullerton, CA, USA) equipped with an argon laser (488 nm) and EXPO 32 software (Beckman Coulter). For each sample 10,000 events were acquired. For analysis of topo IIα expression, a gate was set on the PI-histogram to exclude sub-G0/G1 events and then a cut-off line was set on the isotype control (Fig. 1). The proportion of topo IIα negative cells in the surviving cell population was then calculated and used for further analysis.

Statistical analysis. Differences in the proportions of topo IIα negative cells were tested by the Wilcoxon signed rank test (Statistix®8, Analytical Software, Tallahassee, FL, USA). To analyse the effect of increasing drug concentrations on the proportion of topo IIα negative cells an extended regression model was used. The model was formulated to handle correlations between consecutive observations on the same patient (i.e. intra-individual correlation) and allow for substantial inter-individual variation with respect to baseline values. A linear regression was designed with individual specific intercepts and an assumed fixed slope for all individuals to fulfil these criteria. The regression model used was, 'proportion of topo IIα negative cells = α_i + ß drug exposure', with i denoting the i:th individual. Estimation of model parameters was done as a mixed model in the statistical software SAS (version 9.1, Cary, NC, USA).

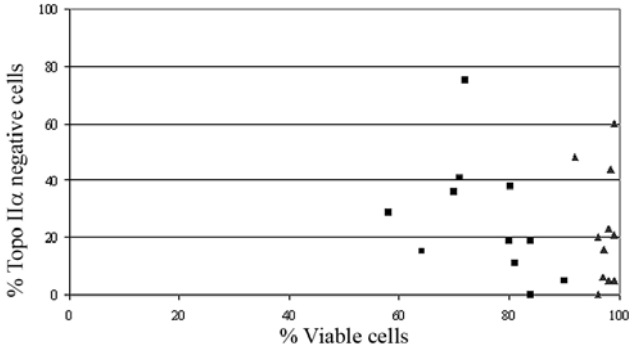

Figure 2. Topo IIα expression in viable human leukaemia cells after 0 (n=11) and 24 h (n=11) of culture. ▲, 0 h; ■, 24 h. Ten patients were examined at both time points.

Results

Cell viability. The viabilities of mononuclear AML cells isolated from 11 of 12 patients are presented in Table I. One case was excluded due to poor cell viability, i.e. the cells were 31% viable in untreated control. After 24 h of culture, the mean cell survival was 76% in the control compared to 31 and 69% after culture with the highest concentrations of daunorubicin and ara-C, respectively.

Expression of topo IIα in control cells. Fig. 2 illustrates the number of topo IIα negative cells relative to cell viability before and after cell culture. The mean fraction of topo IIα negative cells was 25% in freshly prepared cells and 29% after culture for 24 h, which was not statistically significantly different.

Proportion of topo IIα negative cells after drug exposure. Exposure to increasing concentrations of daunorubicin resulted in increased cell death (Table I) and an increase in the fraction of topo IIα negative cells in the viable population (Fig. 3). After exposure to the highest concentration of daunorubicin the mean fraction of topo IIα negative cells in the viable cells was 57% (range: 9-88%). To analyse the expression of topo IIα relative to incubation concentrations a regression model was used (Fig. 3). Treatment with increasing concentrations of daunorubicin resulted in a statistically significant increase in topo IIα negative cells (p=0.0023).

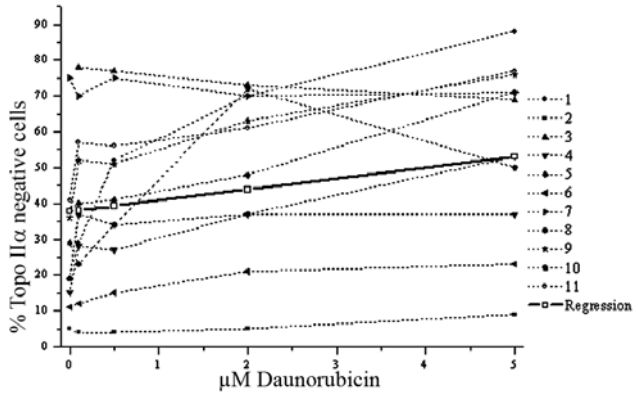

Figure 3. The proportion of topo IIα negative human leukaemia cells in the surviving cell population after exposure to daunorubicin in cell culture. The regression line shows a significantly increased proportion of topo IIα negative cells (Y = 37.7048 + 3.0620 X, p=0.0023).

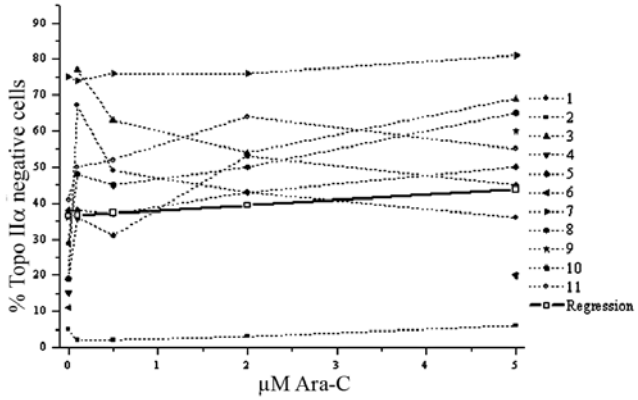

Figure 4. The proportion of topo IIα negative human leukaemia cells in the surviving cell population after exposure to ara-C in cell culture. The regression line shows a positive slope but no significant change in the proportion of topo IIα negative cells (Y = 36.53 + 1.4495 X, p=0.21).

Treatment with 5 μM ara-C resulted in a statistically significant increase in the topo IIα negative fraction compared to untreated control cells (p=0.0059). However, when using the regression model to analyse the variation of expression in relation to increasing incubation concentrations this increase was not statistically significant (Fig. 4).

Discussion

Topo IIα is an essential nuclear enzyme that is normally expressed in proliferating cells. Although the enzyme is highly likely to be important for the effect of several cytostatic drugs used in treatment of AML, no study has demonstrated that the expression of topo IIα is a prognostic factor (10-13). Nonetheless, a low expression of topo IIα could still play a key role in resistance to daunorubicin. Exposure of leukaemia cells to increasing concentrations of cytostatic drugs *in vitro* could facilitate the selection of a resistant subpopulation that can be further analysed. In the present study, we used this approach to analyse the expression of topo IIα in leukaemia cells after exposure to increasing concentrations of daunorubicin or ara-C.

Inter-individual variation in the expression of topo IIα was observed in the present study. This variation was expected due to observations of inter-individual variation in earlier studies (8,14). Considering the variation in the topo IIα expression the method for the statistical analysis was chosen to allow for this variation and at the same time handle intra-individual correlations.

Analysis of the cell cycle distribution in fresh mononuclear cells revealed that most cells were in the G0/G1 phase (data not shown), with a high percentage positive for topo IIα indicating that the cells were actively in the cell cycle. During culture of leukaemia cells it is known that the proliferation rate decreases and that spontaneous cell death occurs. Therefore, one would expect a decreased level of topo IIα in untreated control cells after 24 h of culture in our study. However, even though all the cases included in the present study showed a decrease in the amount of viable cells in the untreated controls after culturing, the expression of topo IIα was clearly not correlated with cell death.

In the present study, the drug concentrations were chosen to range from below to higher than what is achieved when treating patients. Incubation with 0.2 μM daunorubicin for 1 h and continuous exposure to 0.5 μM ara-C results in the same intracellular concentration as 1 h infusion of 60 mg/m^2 daunorubicin and a continuous infusion of 100 mg/m^2 ara-C (15). However, with continuous ara-C incubation, cell death *in vitro* is not apparent until after more than two days. As a consequence of this, the higher cell survival after incubation with ara-C compared to daunorubicin was expected.

Incubation of AML cells with increasing concentrations of daunorubicin resulted in an increased proportion of topo IIα negative cells compared to untreated cells. In contrast, incubation with ara-C resulted in an increase that was not as prominent and only statistically significant at the highest treatment concentration. The expression of topo IIα has been shown to be regulated at the mRNA level (16,17). The stability of topo IIα mRNA is known to change during the cell cycle, with a half-life time of 30 min in the G1 phase compared to 4 h in the S phase (16). Exposure of AML cells to daunorubicin or ara-C *in vitro* is likely to result in cell stress even if 24 h of culture is not enough time to cause cell death of all cells. In unfavourable conditions, p53 will cause cell cycle arrest and thereby inhibit cell proliferation. This could be one of the underlying mechanisms for the increased proportion of topo IIα negative cells, where cell cycle arrest in the G1 phase indirectly results in lower levels of topo IIα. It has also been shown that p53 regulates the transcription of topo IIα, which can prevent cells entering the S phase. In our study, it is likely that the decreased expression of topo IIα following daunorubicin or ara-C exposure is explained, in part, by cell cycle arrest.

Another possible explanation for the increased proportion of topo IIα negative cells is that the treatment resulted in the selection of topo IIα negative cells, since these cells should theoretically be more resistant to therapy. In our study this mechanism could have occurred following incubation with daunorubicin, which exerts its cytotoxic effect via topo IIα. With the design of our study it is not possible to differentiate such selection from a down-regulation of topo IIα caused by cell stress or cell cycle arrest. However, we also observed a

small increase in topo IIα negative cells following exposure to ara-C, which according to present knowledge cannot be explained by selection.

In conclusion, our results show that incubation with daunorubicin at increasing concentrations causes an increased fraction of topo IIα negative cells. The mechanism for this is either cell cycle arrest or a selection of pre-existing topo IIα negative subpopulations. Regardless of the mechanism, an increased fraction of topo IIα negative cells can theoretically contribute to a reduced effect of the second dose of daunorubicin. The clinical relevance of these interactions can be further elucidated in experimental and clinical studies.

Acknowledgements

This study was supported by grants from the Örebro County Research Committee and the Swedish Cancer Society.

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