

Rapid Induction of P-Glycoprotein mRNA and Protein Expression by Cytarabine in HL-60 Cells

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Abstract. *Background: Overexpression of P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and glutathione-S-transferase π (GST π) is associated with drug resistance in acute myeloid leukemia (AML). The short-term effects of drug exposure on their expression levels were investigated. Materials and Methods: HL-60 cells and drug-resistant sublines were cultured with or without daunorubicin (DNR) and cytarabine (Ara-C). At several time-points the expression levels of P-gp, BCRP and GST π were determined. Results: After exposure to Ara-C, P-gp mRNA rapidly increased in all the cell lines. P-gp protein was detected in the sensitive cells after 8 h exposure to Ara-C. GST π mRNA increased in the resistant cells, but no change in BCRP mRNA was observed. Exposure to DNR revealed rapidly increased P-gp and GST π mRNA in the resistant cells. Conclusion: Ara-C rapidly increases P-gp mRNA and protein expression in sensitive and resistant cells, and GST π mRNA in resistant cells, *in vitro*. This may be of clinical importance during AML induction chemotherapy.*

The long-term prognosis following chemotherapy in acute myeloid leukemia (AML) is generally poor (1). This is mainly due to drug resistance, which is displayed by a significant number of patients who do not respond to initial chemotherapy and by patients who relapse after an initial response (2). At relapse, resistance to further chemotherapy is often clinically evident (3). There are several putative mechanisms of drug resistance including drug efflux by membrane pumps of the ATP-binding cassette superfamily, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), or drug metabolism by cytosolic enzymes,

such as glutathione-S-transferase π (GST π ; GSTP1) (4, 5). Membrane pumps have been extensively studied, especially P-gp, which is a 170 kDa protein encoded by the multidrug resistance (ABCB1) gene on chromosome 7 (6). A variety of chemical compounds are substrates for this efflux pump, which has been associated with the multidrug resistance phenotype (7, 8). Standard treatment of AML includes the anthracycline daunorubicin (DNR), which is a P-gp substrate, and the nucleoside analogue cytarabine (Ara-C), which is not a P-gp substrate (9). Over-expression of P-gp at diagnosis has been shown to be an independent negative prognostic marker in AML (10-12), while attempts to improve treatment results by adding P-gp inhibitors to standard chemotherapy have shown conflicting but mainly disappointing results (13-17).

The emergence of drug resistance during long-term chemotherapy is believed to be due to the selection or induction of the multidrug resistance phenotype, and/or clonal development during long-term chemotherapy, although higher expression levels of P-gp at relapse compared to P-gp expression levels at diagnosis have not been consistently proven (16, 18). P-gp expression in drug-sensitive leukemic cell lines can be induced by long-term exposure to cytostatic drugs (19). The short-term effect of cytotoxic drugs *in vitro* on P-gp expression has also been studied to some extent. For instance, P-gp substrates, as well as non-P-gp substrates, have been shown to induce P-gp mRNA and P-gp expression in human leukemic cell lines (20). Furthermore, P-gp expression can be induced as early as within 4-8 h of exposure to anthracyclines (21-23). In samples from patients with AML it has been demonstrated that P-gp protein expression and function can be induced *in vitro* 16 h post-initiation of drug exposure, including exposure to Ara-C (24).

The early induction of drug resistance in leukemic cells may be of clinical importance. The aim of this study was to further investigate the effect of DNR and Ara-C, in clinically relevant concentrations, on P-gp, BCRP and GST π expression in leukemic cells that exhibit different basal levels of drug resistance.

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Key Words: Drug resistance, P-glycoprotein, daunorubicin, cytarabine, HL-60 cells, glutathione-S-transferase π .

Materials and Methods

Cell lines and culture conditions. Human leukemic HL-60 cells (American type culture collection (ATCC), Rockville, MD, USA), sensitive to doxorubicin (HL-60 S) and the sublines HL-60 R0.5 and HL-60 R5, resistant to 0.5 μM and 5 μM doxorubicin, respectively (19), were grown in suspension in RPMI-1640 medium containing 10% fetal bovine serum and 2.5% 1 M HEPES (all from Gibco, Paisley, Scotland, UK). The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. The resistant cells were maintained without drug for two weeks prior to each experiment. The sublines HL-60 R0.5 and R5 have previously been shown to express high levels of P-gp, while HL-60 S has been shown to be negative for P-gp. Compared to HL-60 S cells, the IC₅₀ of DNR is 80-fold higher for HL-60 R0.5 cells and 858-fold higher for HL-60 R5 cells. The IC₅₀ of Ara-C has been shown to be 6.5-fold higher for HL-60 R0.5 cells and 2.1-fold higher for HL-60 R5 cells compared to HL-60 S cells (19). *ABCB1*, *GSTP1* and *BCRP* mRNA expression levels were determined in all three cell lines by real-time RT-PCR. P-gp was further investigated at the protein level by Western blot and flow cytometry.

Incubations. The cells were suspended in pre-heated medium (37°C) in culturing flasks, at a concentration of 5×10^5 cells/ml and a final volume of 8 ml. Each cell line was exposed to a final concentration of either 0.2 μM DNR (*i.e.* Cerubidin from Aventis Pharma, Bromma, Sweden), 0.5 μM Ara-C (*i.e.* Cytarabine from Pfizer AB, Sollentuna, Sweden) or RPMI 1640 as a negative control, in three separate series. These concentrations were chosen to achieve clinically relevant exposure (25). From each series, samples were collected before exposure, immediately at exposure time 0 and at 10 and 30 min, as well as 1, 2, 4, 8, 12, 16, 24 and 36 h, for real-time RT-PCR and Western blot analysis. The reaction was stopped with 10 ml of ice-cold RPMI-1640, after which the samples were pelleted at 400 $\times g$ for 5 min at 4°C and the supernatant was discarded. For flow cytometry analysis of protein expression, samples were collected from each series before exposure and after 4, 8, 12 and 24 h of exposure to DNR or Ara-C. The samples were immediately pelleted at 400 $\times g$ for 5 min at room temperature and the supernatant was discarded.

RNA preparation and cDNA synthesis. The total RNA was isolated with an RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The total RNA concentration and the quality of the isolated RNA were assayed with an Agilent Bioanalyzer 2100 and RNA 6000 Nano Assay Kit (Agilent Technology, Santa Clara, USA) according to the manufacturer's protocol. To determine the quality of the RNA the ratio of 28S and 18S generated using the same method was used. A ratio above 1.5 indicated that the RNA was considered of acceptable quality for use in subsequent analyses. For first-strand cDNA synthesis, an Omniscript® Reverse Transcription kit (Qiagen) was used according to the manufacturer's instructions. Briefly, 1 μg of the total RNA was used to produce first-strand cDNA with oligo(dT)_n.

Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR). Real-time RT-PCR was performed using the thermal cycler TaqMan 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 7500 Fast Sequence Detection and Relative Quantification software packages. The PCR

cycling conditions were, step one: 95°C for 2 min and step two: 95°C for 3 s and 60°C for 30 s (step two repeated 40 times). The combined primers and probes were purchased from Applied Biosystems (Stockholm, Sweden). Hs00184491_m1 was used to detect *ABCB1*, Hs00184979_m1 was used to detect *BCRP* and Hs02512067_s1 was used to detect *GSTP1*. As an internal control 18S (Hs99999901_s1) was used. The PCR reactions were performed in 20 μl using the TaqMan Universal PCR Master Mix (Applied Biosystems, Sweden) and 2 μl cDNA. The experiments were carried out in duplicate and the mean values were used for the analysis.

Western blot. The pelleted cells were lysed in 10 mM Tris/HCl lysis buffer with 150 mM sodium chloride, 1% Nonidet P-40, 1 mM EDTA, 50 mM sodium fluoride, 40 mM β -glycerophosphate, 1 mM sodium orthovanadate and protease inhibitors, *i.e.* phenylmethylsulphonyl fluoride, leupeptin, pepstatin and aprotinin (all diluted to a final concentration of 1 $\mu\text{g}/\text{ml}$), at 4°C for 10 min and stored at -80°C in cryo-preservation tubes (Sarstedt AG and Co, Nümbrecht, Germany). The protein concentrations were determined by DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) at an absorbance of 750 nm on a Multiscan Ascent (Thermo Labsystems, Stockholm, Sweden). Twenty μg of protein lysate that was diluted 2:1 in urea buffer (8 M urea, 170 mM SDS, 0.5 mM bromophenol blue, 455 mM dithiothreitol, 50 mM Tris (pH 6.8 adjusted with HCl)), as well as a molecular weight marker (Precision Plus Protein Standards Dual Color, Bio-Rad), were separated on 7% SDS gels at 140 V for 1 h. After electrophoresis, the proteins were transferred to polyvinylidene fluoride transfer membranes at 30 V overnight. Nonspecific binding was blocked by 5% bovine serum albumin (Roche Diagnostics GmbH, Indianapolis, IN, USA) in TBS buffer. The membranes were incubated with P-gp primary mouse monoclonal antibody (C219), (Abcam, Cambridge, UK), diluted 1:500 in TBS containing 0.1% Tween (TBS-T) (BioRad, Stockholm, Sweden), at 4°C overnight. The blots were washed thoroughly in TBS-T and incubated in secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (C1607) at a dilution 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. After a final wash in TBS-T, HRP was detected using the Immuno-Star HRP Substrate Kit from Bio-Rad and a Molecular Imager® ChemiDoc™ XRS, as described in the manufacturer's instructions.

Flow cytometry. The pelleted cells were incubated with FITC-conjugated anti-P-gp (clone 17F9) and 7-AAD (BD Biosciences, Stockholm, Sweden) for 10 min at room temperature in the dark. The cells were then resuspended in 400 μl of PBS and analyzed immediately using an EPICS® ALTRA™ (Beckman Coulter, Fullerton CA, USA) flow cytometer, equipped with an Argon laser (488 nm) and Expo™ 32 software (Beckman Coulter). The P-gp expression in the drug-exposed cells was determined as the mean fluorescence intensity in relation to the P-gp expression in cells cultured in RPMI only at each point of time. The flow cytometry analyses were performed in duplicate for each sample and the mean value of the duplicates was used for the analysis.

Results

Real-time RT-PCR. In the HL-60 S cells exposure to Ara-C for 10 min resulted in an increase in *ABCB1* mRNA expression to 1.7-fold compared to the cells cultured in RPMI alone and remained unchanged throughout 36 h of

incubation. No rapid increase in *ABCB1* mRNA expression was observed in the HL-60 S cells after incubation with DNR, but a 1.6-fold increase was observed after 24 h. For *GSTP1* mRNA, incubation with DNR showed a trend towards increased levels from the initiation of treatment and after 12 h a 1.8-fold increase was seen. After incubation with Ara-C a minor decrease in the *GSTP1* mRNA level was seen after 10 min incubation. Only minor changes were seen also at the following time-points. Culturing of the HL-60 R0.5 cells in RPMI revealed a spontaneous increase in *ABCB1* mRNA expression over time. Compared to that, a 3.1-fold increase in *ABCB1* mRNA expression was detected after 10 min exposure to Ara-C. This increased expression declined and after 2 h no increase above the level in RPMI alone was seen. After incubation with DNR for 10 min, a 2.8-fold increase in the *ABCB1* mRNA expression, as compared to the cells cultured in RPMI, was observed after which the expression declined. After 4 h there was no increase compared to the cells cultured in RPMI alone. Exposure to DNR resulted in a 2.4-fold increase in *GSTP1* mRNA after 10 min compared to RPMI alone and the increased levels remained throughout the incubation time. A 3.4-fold increase in *GSTP1* mRNA was observed after 10 min exposure to Ara-C. This increase declined during the incubation time and was undetectable after 36 h.

In the HL-60 R5 cells after incubation with Ara-C for 10 min, a 1.9-fold increase in *ABCB1* mRNA expression was observed. Following a period of decline the expression further increased to 2.4-fold at 12 h and 4.5-fold at 36 h. Only negligible changes in *ABCB1* mRNA expression were observed at all the time-points during the 36 h of incubation with DNR compared to cells cultured in RPMI alone. Exposure to DNR resulted in a negligible increase in *GSTP1* mRNA after 10 min, but after 1 h of exposure the levels were 1.5-fold higher than in the cells cultured in RPMI alone and further increased during the incubation time to a maximum of 4.0-fold after 36 h. After 10 min exposure to Ara-C there was a 4.0-fold increase in *GSTP1* mRNA. This increase remained and further increased to 5.8-fold after 36 h. *BCRP* mRNA was undetectable in all three cell lines, regardless of exposure time, throughout the experiment.

Western blot. In the HL-60 S cells exposed to Ara-C, P-gp expression was detectable after 8 h treatment, while no P-gp expression was detected during 36 h exposure to DNR. The high levels of P-gp expressed by the HL-60 R0.5 and HL-60 R5 cells from the initiation of treatment, did not change throughout the entire 36 h of exposure to Ara-C or DNR. A spontaneous increase in the P-gp expression level was seen continuously for 24 h, in the HL-60 R0.5 cells cultured in RPMI.

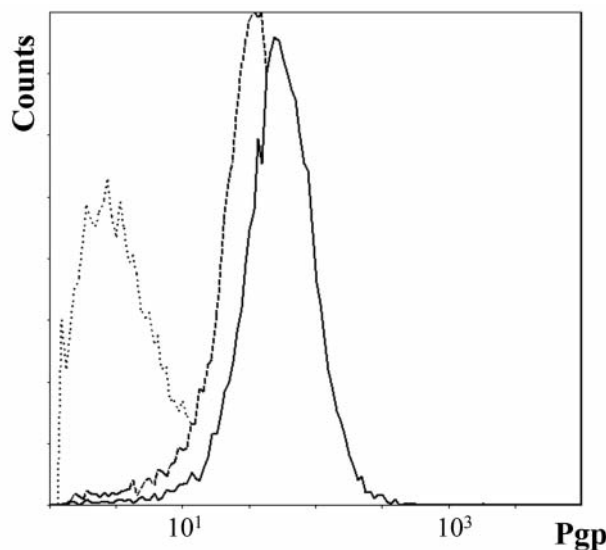


Figure 1. *Pgp* expression in HL-60 S (dotted line), HL-60 R0.5 (broken line) and HL-60 R5 (continuous line) cells, determined by flow cytometry.

Flow cytometry. For the HL-60 S cells, all the results were adjusted to viable cells only. Cell death in the HL-60 R0.5 and HL-60 R5 cells was negligible during the 24 h of incubation. Untreated HL-60 S cells did not display any detectable P-gp, while the untreated HL-60 R0.5 and HL-60 R5 cells expressed 1.9-fold and 3.2-fold increased levels of P-gp, respectively, compared to HL-60 S cells (Figure 1). In the HL-60 S cells exposed to Ara-C or DNR, no increase in P-gp expression was detected during the 24 h of incubation. In the HL-60 R0.5 cells exposed to Ara-C, a 1.2-fold increase in P-gp expression was seen after 24 h compared to cells cultured in RPMI alone. There was no increase in P-gp expression in the cells exposed to DNR (Figure 2). In the HL-60 R5 cells a 1.3-fold increase in P-gp expression was found after 24 h of exposure to Ara-C (Figure 3), while there was no increase detected in the cells exposed to DNR.

Discussion

A rapid increase in the mRNA expression level of *ABCB1* was found and an increase in P-gp protein expression in sensitive and resistant myeloid leukemic cells after exposure to Ara-C was verified. In addition, a rapid increase in the expression level of *GSTP1* mRNA in the resistant, but not the sensitive cells was found.

The increase in *ABCB1* mRNA expression after exposure to 0.5 μ M Ara-C was evident after 10 min, in all the cell lines. On the other hand, 10 min exposure to 0.2 μ M DNR showed rapid up-regulation of *ABCB1* mRNA only in the HL-60 R0.5 cells. Such up-regulation by short-term exposure

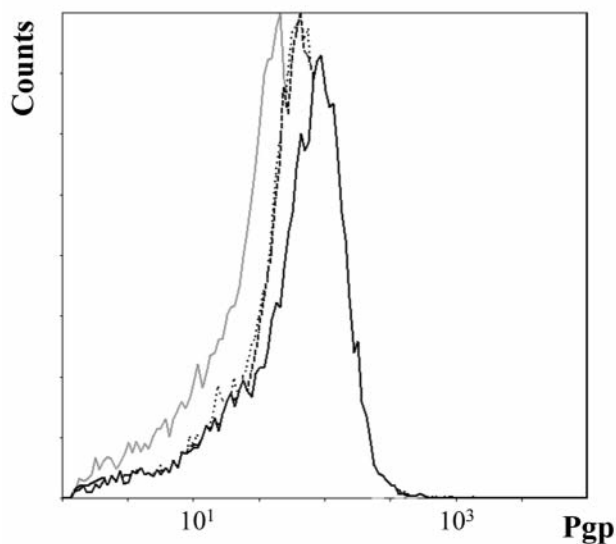


Figure 2. *Pgp* expression determined by flow cytometry in the HL-60 R0.5 cells before treatment initiation (grey line), after 24 h culture in RPMI alone (dotted line), after 24 h treatment with DNR (broken line) and after 24 h treatment with Ara-C (continuous line).

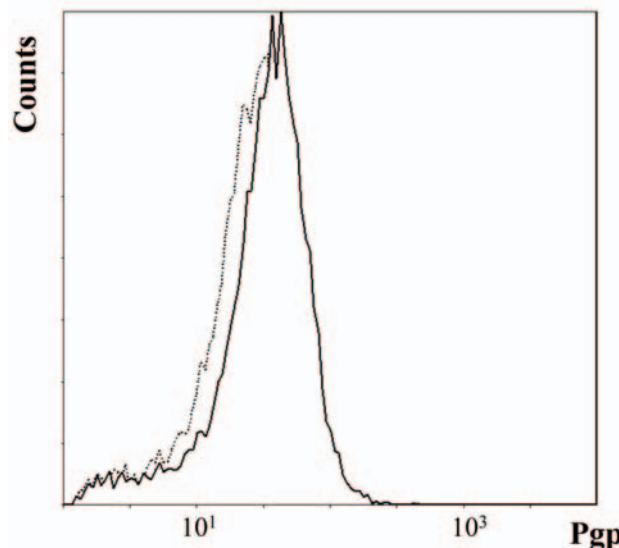


Figure 3. *Pgp* expression determined by flow cytometry in the HL-60 R5 cells after 24 h culture in RPMI alone (dotted line) and after 24 h treatment with Ara-C (continuous line).

to cytostatic drugs has been demonstrated previously in human non-myeloid cells (20-23, 26). As an example, Abolhoda *et al.* exposed solid human tumor sarcoma cells to doxorubicin *in vivo* and demonstrated up-regulated *ABCB1* mRNA after 50 min (26).

In addition, detectable P-gp protein was found by Western blot in the HL-60 S cells after 8 h exposure to Ara-C. In the HL-60 R0.5 and R5 cells, P-gp expression was detectable prior to incubation with Ara-C and no obvious increase over the incubation period could be detected. Using flow cytometry to detect P-gp a 1.2-1.3-fold increase in P-gp expression after 24 h exposure to Ara-C was found in both the drug resistant sublines. These results should be compared to the initial 1.9-fold difference in P-gp protein expression between HL-60 S and HL-60 R0.5, which corresponds to a 80-fold difference in the degree of drug resistance (19). This suggests that a small increase in P-gp protein expression, as detected by flow cytometry, may result in a major increase in the degree of drug resistance.

In a recent study, Hu *et al.* determined P-gp expression and function by flow cytometry in myeloid leukemia patient samples exposed *in vitro* to DNR or Ara-C. An up-regulation in P-gp expression, which correlated well with P-gp function, was evident after 16 h of drug exposure in resistant as well as sensitive cells (24). However, a spontaneous increase in both *ABCB1* mRNA and protein expression levels in the HL-60 R0.5 cells cultured in RPMI was found in the present study. Spontaneous increase in *ABCB1* mRNA has previously been observed in cultured

human cells (27, 28). Therefore, it is crucial to subtract the spontaneous increase of P-gp when estimating both mRNA and protein levels. After such correction, no increased P-gp protein expression induced by DNR treatment was detected in the present study in the resistant cell lines. Hu *et al.* did not make these corrections, which may have resulted in the higher mean fluorescence intensity for P-gp exposed to DNR or Ara-C (24). In the same study Hu *et al.* demonstrated an increased P-gp protein expression *in vivo* after a simultaneous exposure to DNR and Ara-C (24). This increase in P-gp expression correlated well to the present flow cytometric results *in vitro* after subtracting the spontaneous increase. According to the present results one could therefore speculate that the major increase in P-gp expression *in vivo* was due to the short-term exposure to Ara-C rather than DNR.

In this study all three cell lines were negative for *BCRP* mRNA regardless of drug exposure. Regarding *GSTII* a marked increase in *GSTP1* mRNA levels was found after short-term incubation with Ara-C in both the HL-60 R0.5 and R5 cells, while no increase was observed in the HL-60 S cells. After 10 min incubation with DNR, an increase in *GSTP1* mRNA was found in the HL-60 R0.5 cells, but not in the R5 cells, while an increase was first seen after 12 h in the HL-60 S cells. Based on a previous finding that *GSTπ* is an inhibitor of c-Jun N-terminal kinase 1 (JNK1), it has been suggested that elevated levels of *GSTπ* might have an anti-apoptotic effect that is mediated *via* JNK1, which is involved in apoptotic signaling *via* the mitogen-

activated protein kinase pathway (29, 30). This supports the standpoint that GST π -over-expressing cells are resistant to non-GST π substrates such as DNR and Ara-C, *i.e.* these cells are not resistant to specific drugs but to apoptosis induced by various drugs (31). The present results may therefore reflect a general response to stress, *e.g.* exposure to cytostatic drugs, in leukemic cells, making them less prone to apoptosis.

With respect to the induction of *ABCB1*, one could speculate that Ara-C given before, or concomitantly with DNR or other P-gp substrates, might reduce the clinical effects of these drugs. If so, that could be of importance for the sequential scheduling of anthracyclines and Ara-C during induction chemotherapy for AML. If this is applicable to the *in vivo* situation during induction therapy remains to be determined in further studies.

In conclusion, induction of *ABCB1*, more rapidly than previously shown, in myeloid leukemic cell lines by short-term exposure to Ara-C *in vitro* is verified. Rapid induction of *GSTP1* mRNA also occurs in the resistant sublines HL-60 R0.5 and R5, after drug exposure.

Acknowledgements

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

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Received April 20, 2009

Revised June 29, 2009

Accepted July 16, 2009