A low but functionally significant MDR1 expression protects primitive haemopoietic progenitor cells from anthracycline toxicity

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Summary. Pgp is expressed on normal haemopoietic progenitor cells. The significance of the efflux pump in protecting normal progenitors for anthracycline toxicity is not defined and is the subject of this study. Pgp was measured in CD34+ progenitors with a rhodamine efflux assay. A high efflux, modulated by verapamil, was only found in a distinct subpopulation (20-30%). Pgp measured by the monoclonal antibody (MoAb) MRK-16 was low in the rhodamine dull, but significantly (P<0.04) higher than in the rhodamine bright cells. Reverse transcriptase polymerase chain reaction (RT-PCR) of MDR1 mRNA showed a very weak signal in both populations. In a single-cell clonogenic assay, rhodamine dull cells appeared less sensitive to anthracyclines (IC50 daunorubicin 0.005µg/ml; adriamycin 0.01µg/ml) compared to rhodamine bright cells (IC50 daunorubicin 0.0025µg/ml; adriamycin 0.01µg/ml). Furthermore, verapamil significantly (P<0.05) potentiated anthracycline toxicity only in the rhodamine dull cells, proving its Pgp-specific modulating effect. Rhodamine dull cells gave larger and more mixed colonies compatible with a more primitive origin. Although detection with MoAbs and RT-PCR revealed a low Pgp level, functionally this Pgp appeared to be very important in protecting primitive progenitors against anthracycline toxicity. This protection can be jeopardized by administration of Pgp modulators.

Keywords: anthracyclines, haemopoietic progenitors, MDR1, P-glycoprotein, rhodamine.

Treatment of cancer with chemotherapy may fail due to selection for or induction of resistance mechanisms. Haematological and non-haematological toxicity limits further dose intensification. In the last decade, much research focused on a common form of multidrug resistance, P-glycoprotein-170 (Pgp) expression. Pgp is a transmembrane-located glycoprotein, encoded by the Multi Drug Resistance 1 (MDR1) gene (Ueda et al, 1986). Pgp functions as an energy-dependent efflux pump which transports many structurally unrelated compounds such as vinca alkaloids, taxols, actinomycin D, epipodophyllotoxins, mitoxantrone and anthracyclines (Ling, 1992). Several agents, e.g. verapamil, are effective modulators of this efflux pump (Slater et al, 1986; Hofmann et al, 1992; Weaver et al, 1993; Boesch & Loor, 1994).

In vitro experiments with cell lines showed a strong correlation between Pgp expression and rhodamine retention. The efflux assay with the fluorescent dye rhodamine is a sensitive method to assess functional MDR1 expression (Neyfakh, 1988; Ellerth et al, 1989; Ludescher et al, 1992). Other methods like PCR of the MDR1 messenger RNA or immunostaining with MoAbs for different epitopes (4E3, C219, JSB1, MRK16) have also been applied in order to determine Pgp expression (Murphy et al, 1990; Noonan et al, 1990; Schinkel et al, 1991; Krishan et al, 1991; Van Acker et al, 1993). Conflicting results have been reported using different methods (Zhou et al, 1992; Brophy et al, 1994; Nussler et al, 1994; Bailly et al, 1995).

Pgp is not only present on malignant cells but also in various normal tissues such as blood-brain barrier, jejunum, colon, kidney, adrenal gland, biliary and pancreatic ductules (Sugawara et al, 1988; Cordón Cardo et al, 1989; van der Valk et al, 1990), peripheral blood cells (fractions of T and B cells) (Neyfakh et al, 1989; Chaudhary et al, 1992; Gupta et al, 1992; Klimecki et al, 1994) and on haemopoietic progenitor cells (Drach et al, 1992; Marie et al, 1992;...
Hegewisch & Becker, 1993; Licht et al., 1994). Chaudhary & Roninson (1991) showed, by double staining with rhodamine and MRK16, an inverse correlation between rhodamine retention and Pgp expression on normal human bone marrow cells. A low rhodamine retention also corresponded with a high CD34 expression and high clonogenic capacity of these cells.

Little is known about the physiological function of Pgp on haemopoietic progenitors and its significance in protecting haemopoietic cells during therapy with cytotoxic drugs. The application of Pgp modulators in an attempt to overcome drug resistance of malignant cells may be nullified by increased toxicity to normal haemopoietic progenitors.

In this study, Pgp expression and function were studied in normal CD34+ cells. A high functional Pgp was measured in 20–30% of the CD34+ cells as indicated by a low retention of rhodamine (dull). In a single-cell conjugation assay these rhodamine dull cells proved to be protected against in vivo attainable anthracycline concentrations and more primitive progenitors in contrast to rhodamine bright cells. Moreover, only in the rhodamine dull cells was retention and toxicity significantly increased by the modulator verapamil.

**MATERIALS AND METHODS**

*Isolation of CD34+ cells.* After obtaining informed consent, bone marrow was collected from healthy donors undergoing bone marrow harvest for allogeneic bone marrow transplantation. Mononuclear cells were isolated by Ficoll-Paque 1·077 g/ml (Pharmacia Biotech, Uppsala, Sweden) density centrifugation. The interphase cells were collected and washed twice with glucose-phosphate buffered saline supplemented with 0·5% w/v bovine serum albumin (BSA), Fraction V, A-9418, Sigma Chemical Co., St Louis, Mo., U.S.A.) (G-PBS-BSA). CD34+ cells were isolated with directly conjugated CD34 antibody-coupled immunomagnetic beads (M-450, coated with '561' a class III epitope anti-CD34, Dynal, Oslo, Norway) using the Dynal protocol. Purity of the cells was controlled by labelling with the MoAb HPCA2 (Becton Dickinson (BD) BV, Etten-Leur, The Netherlands). Cells incubated with IgG1-FITC (Dako, Glostrup, Denmark) served as control. After washing with G-PBS-BSA, cells were resuspended in Iscove’s Medium (Life Technologies, Paisley, Scotland), supplemented with 5% v/v heatinactivated fetal calf serum (FCS, Hyclone, Logan, Utah, U.S.A.) and 10% v/v dimethylsulphoxide (DMSO, Merck-Schuchardt, Munich, Germany) at a concentration of 0·2–0·4×10^6 cells/ml and cryopreserved in 1 ml vials in liquid nitrogen with a temperature controlled freezer (Kryo 10, Planer Products, Sunbury-on-Thames, U.K.). Prior to the experiments, cells were thawed rapidly in a 37°C water bath and diluted in FCS, containing 0·02 mg/ml deoxyribonucleic acid (DNAse from bovine pancreas, Boehringer-Mannheim, Germany), 4 mm MgSO4 and 15 U/ml preserved free heparin. After washing with G-PBS-BSA the cells were resuspended in Iscove’s medium with 10% v/v FCS.

*Efflux assay.* Rhodamine (Rh123, Sigma Chemical Co., St Louis, Mo., U.S.A.) was dissolved in distilled water (dH2O) and stored as a sterile stock solution (10 µg/ml). The cells were incubated (2×10^5 cells/ml) in Iscove’s medium with a final rhodamine concentration of 0·1 µg/ml (60 min, 37°C). After centrifugation, cells were resuspended in dye-free Iscove’s medium with or without 10 µM verapamil (Knoll AG, Ludwigshaven, Germany) and incubated at 37°C to allow the efflux of the fluorochrome by Pgp. To study time dependency of rhodamine uptake and efflux, samples were evaluated at various intervals. A time course of 2 h efflux was standardized to study maximal differences in functional Pgp expression on haemopoietic progenitors.

As anthracyclines are actively pumped out of the cell by Pgp, the efflux assay performed with autoluminescent anthracyclines should yield comparable results. Daunorubicin (Rhone-Poulenc Rorer BV, Amstelveen, The Netherlands) was dissolved in phosphate buffered saline (PBS) (NPBI BV, Emmer-Comsacum, The Netherlands) and stored at −20°C as a sterile stock (50 µg/ml). After loading the cells with 2 µg/ml daunorubicin (60 min, 37°C), the same efflux procedure was applied. Cellular rhodamine and daunorubicin content were measured by flow cytometry.

*Flow cytometric measurement of dye content and single-cell sorting.* For the measurement of cellular dye content as well as single-cell sorting, a Coulter Epics Elite Flow cytometer, equipped with an autoclone device (Coulter, Miami, Florida) was used. Cells were excited with a single Argon ion laser emitting at 488 nm, running at 15 mW (standard setting). Gating on forward and right angle scatter was used to exclude dead cells and debris. Fluorescence intensity of cellular rhodamine and daunorubicin were measured using respectively a 515 nm and a 550 nm long pass filter. After 2 h efflux, 25% of the cells with the highest (bright) or lowest (dull) rhodamine content were sorted to elucidate differences between cells with respectively a relatively low or high functional Pgp expression.

*Rhodamine uptake assay.* After sorting rhodamine dull and bright CD34+ cells, a second incubation (without efflux) with 0·1 µg/ml rhodamine (60 min, 37°C) (2×10^5 cells/0·5 ml Iscove’s medium) in the presence or absence of 10 µM verapamil was performed. After 60 min incubation, rhodamine retention was at a steady state, as a result of an equilibrium between uptake and efflux. As verapamil blocks the Pgp dependent efflux, differences in rhodamine content in the presence and absence of verapamil affirmed functional Pgp expression.

*Immunostaining for Pgp.* For immunostaining of Pgp, two different MoAbs were applied: C219 (Centocor Corporation, Malver, Pa., U.S.A.) recognizes an internal, and MRK16 (Kamiya Biomedical Company, Thousand Oaks, Calif., U.S.A.) an external Pgp epitope. Before staining with C219, rhodamine dull and bright CD34+ cells were sorted, pelleted and fixed in 1 ml Permeafix (Ortho Diagnostic Systems Inc., Raritan, N.J., U.S.A.) (room temperature, 40 min) followed by 10 min incubation in G-PBS-BSA. After incubation (4°C, 10 min) in 20% v/v pooled human serum/PBS to block nonspecific binding, cells were incubated with 2 µg mouse anti-human C219 (4°C, 30 min), washed with G-PBS-BSA (4°C) and then stained with goat anti-mouse IgG-FITC (DAKO) (4°C, 30 min).

After the rhodamine efflux assay, viable cells were blocked...
for non-specific binding cells. Thereafter, 5 μg mouse anti-human MRK16 was added to the pellet (4°C, 30 min). Next, cells were washed with G-PBS-BSA (4°C) and incubated with goat anti-mouse IgG-PE (DAKO) (4°C, 30 min). After a final wash step with G-PBS-BSA, analysis was performed flow cytometrically (standard setting) using a 550 dichroic mirror with a 525 band pass filter or a 575 band pass filter to measure respectively the FITC and PE signal. The MRK16 signal was measured in subpopulations by gating 25% of the most rhodamine dull and rhodamine bright cells. Appropriate isotype controls (mouse anti-human IgG/IgG2a) were used in the control tubes. The staining procedure was validated on the cell line A2780/dox5.

RNA isolation and RT-PCR of the MDR1 expression. RNA was extracted using the guanidium thiocyanate acid phenol chloroform procedure (Puisant & Houdebine, 1990). The samples were stored at −80°C. The cdNA synthesis reaction was performed in a total volume of 20 μl, containing 50 mM Tris-HCl (pH 8·3), 75 mM KCl, 1·5 mM MgCl2, 10 mM DTT, 625 μM dNTPs, 5 μM random hexamers (Pharmacia, Uppsala, Sweden), 20 U RNAsin (Promega, Madison, Wis., U.S.A.) and 200 U Mo-MLV reverse transcriptase (Life Technologies). To this suspension 5 μl of RNA or dH2O was added, and the mixture was overlaid with 70 μl mineral oil. The RT reaction was performed for 10 min at 20°C, 45 min at 42°C, 10 min at 95°C, and subsequent cooling to 4°C. After cdNA synthesis the total volume was increased up to 100 μl containing 50 mM KCl, 20 mM Tris pH 8·4, 1·5 mM MgCl2, 0·001% gelatin, 0·251 mM dNTPs, 30 pmol of each primer and 2·5 U Taq polymerase (Gibco BRL, Gaithersburg, Murielle Smeets et al.

RESULTS

Dye retention and modulation by verapamil

After 1 h of incubation with rhodamine, all CD34+ cells contained rhodamine (Fig 1). No distinct subpopulation with a different rhodamine content was observed. However, after resuspension in dye-free medium the efflux was heterogenous. A relatively fast efflux was observed only in a subpopulation of the CD34+ cells. Within 2 h efflux, a distinct rhodamine dull subpopulation of 20–30% of the cells with a rhodamine content below the initial uptake could be distinguished. During prolonged exposure the efflux slowed down, but no plateau was observed. After 2 h incubation of rhodamine loaded cells in rhodamine free medium containing verapamil, almost no decrease of rhodamine content was measured (Fig 2).

Table I. Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>MDR1-5</td>
<td>TGC TGG TTG CTG CTT ACA TTC</td>
</tr>
<tr>
<td>MDR1-3</td>
<td>GCC AAA ATC ACA AGG GTT AGC</td>
</tr>
<tr>
<td>MDR1-int</td>
<td>ACA TCA TCT GTA AGT CCG GTG TT</td>
</tr>
<tr>
<td>B2M-5</td>
<td>CTC GCG CTA CTC TCT CCT TCT</td>
</tr>
<tr>
<td>B2M-3</td>
<td>TGT CGG ATT GAT GAA ACC CAG</td>
</tr>
<tr>
<td>B2M-int</td>
<td>CAG GTT TAC TCA CGT CAT CCA GCA GAG AAT</td>
</tr>
</tbody>
</table>

Pgp Modulators increase Haematological Toxicity

Fig 1. Representative example of rhodamine efflux in normal CD34+ cells. After 1 h incubation all cells showed a high rhodamine uptake (rhodamine bright). Rhodamine efflux was observed in all cells. However, a subpopulation of the cells initially showed a relatively fast rhodamine efflux and became rhodamine dull (below the initial uptake). The initial fast efflux decreased gradually with time.

Fig 2. Rhodamine content of normal CD34+ cells after 2 h efflux in dye free medium without (1) and with (2) verapamil. Sorting experiments were performed according to the gate setting A1 (25% of the most rhodamine dull cells) and B1 (25% of the most rhodamine bright cells).
The daunorubicin efflux assay yielded similar results. A comparable fraction (20–30%) of the CD34⁺ cells showed a strong daunorubicin efflux resulting in a low content of daunorubicin (daunorubicin dull). In the presence of verapamil the efflux of daunorubicin was blocked and an increased retention of daunorubicin was observed (Fig 3).

Modulation of rhodamine or daunorubicin efflux in the presence of verapamil strongly suggests the expression of functional Pgp. To affirm the differences in functional Pgp expression between rhodamine dull and bright cells, 25% of the most rhodamine dull and 25% of the most rhodamine bright cells, were sorted. In the presence of verapamil a 2-7-fold higher increase of rhodamine retention was measured in the rhodamine dull cells compared to a 1:1-fold increase in the rhodamine bright cells as shown in Fig 4.

**Immunostaining for Pgp expression**

Staining with the MoAbs C219 against an internal and MRK16 against an external epitope was weak in both the rhodamine dull and bright cells. No significant quantitative differences in Pgp were measured with C219. Staining with MRK16 yielded a significantly \( P < 0.04 \) stronger signal in the rhodamine dull cells compared to the rhodamine bright cells (Table II). Measurement of Pgp with MRK16 on the cell line A2780/dox5 versus A2780/WT showed a much stronger signal (mean fluorescence A2780/dox5/A2780/WT: 8.3).

**RT-PCR of MDR1 mRNA**

The results of southern blotting of the RT-PCR and screening with the MDR-int of rhodamine dull and bright CD34⁺ cells are shown in Fig 5. A weak positive signal for the MDR1-mRNA was visible in both rhodamine dull and rhodamine bright cells, compared to the cell line A2780/dox5. No significant quantitative differences in MDR1 mRNA could be detected between the rhodamine dull and bright cells after correction for the β2M signal. Semi-quantitative analysis was not feasible, as the RT-PCR analysis of CD34⁺ cells was only positive after 35 cycles.
DISCUSSION

To study the functional relevance of Pgp on normal haemopoietic progenitors we used purified CD34+ haemopoietic cells from healthy bone marrow donors. During rhodamine exposure the rhodamine fluorescence was rather homogenous. Only after 2 h of efflux could a distinct subpopulation of 20–30% of the CD34+ cells be characterized as rhodamine dull, suggesting a relatively high Pgp expression. Anthracyclines, also actively effluxed by Pgp, showed comparable results. 20–30% of the cells became daunorubicin dull after 2 h efflux. Both rhodamine and daunorubicin efflux could be completely inhibited by verapamil, which is strongly suggestive for active Pgp function. By forward scatter, the size of rhodamine or daunorubicin bright cells was 2-fold larger compared to the size of rhodamine dull cells. However, differences in rhodamine retention were 4-6-fold. Moreover, differences in efflux and efflux inhibition by verapamil is independent of the cell size. As rhodamine also stains mitochondria, the number and activity of mitochondria may influence rhodamine retention. Darzynkiewicz et al. (1981) showed an increase in rhodamine retention was most probably due to increased mitochondrial binding. We studied the rhodamine dull and bright cells by electron microscopy. No differences in morphology of the cells or number of mitochondria could be detected (data not shown). In addition, staining and efflux studies with daunorubicin not preferentially bound to mitochondria, revealed similar results to rhodamine.

MDR1 expression measured by RT-PCR of the mRNA or staining with the MoAb C219 showed no significant quantitative differences between rhodamine dull and bright cells. Pgp detected by MRK16 was also very weak compared to the MDR1-positive cell line. However, the MRK16 signal was significantly stronger on the rhodamine dull cells versus the rhodamine bright cells in accordance with the observed differences in functional Pgp. Therefore the efflux assay is the most sensitive method to detect small subsets of cells with a high Pgp, remaining obscure by other methods.

The presence of Pgp on haemopoietic progenitors has already been observed by others; however, its functional relevance in protecting haemopoietic cells has not yet been proved. By studying anthracycline toxicity to progenitors with a high and low or negligible Pgp we proved the importance of Pgp on the most primitive progenitors. In a single cell clonogenic assay we showed that the rhodamine dull cells are indeed protected against anthracycline toxicity by its Pgp. The IC50 values of anthracyclines were 2–3-fold higher for the rhodamine dull cells compared to the bright rhodamine bright cells revealed no differences in percentage of colonies of erythrocytes colonies (25% and 21%) or granulocytes/monocytes colonies (63% and 75%). The percentage of mixed colonies grown from rhodamine dull cells (13%) was significantly (P<0.05) higher compared to rhodamine bright cells (<1%) (Table III). Immunostaining with MoAbs of large colonies confirmed the morphological analysis (data not shown).

**Table III. Proliferative capacity of CD34+ cells.**

| Proliferative capacity of rhodamine dull and rhodamine bright cells (% of wells in 96-well plate) |
|---------------------------------------------------------------|---------------------------------------------------------------|
| >50 cells dull/bright | >500 cells dull/bright | >5000 cells dull/bright |
| Mean (%) | 32/25 | 24/13 | 15/3 |
| P value | 0.003 | 0.0002 | 0.0002 |

| Morphology of colonies >50 cells |
|----------------------------------|----------------------------------|
| Granulocytes or monocytes dull/bright | Erythrocytes dull/bright | Mixed dull/bright |
| Mean (%) | 63/75 | 25/21 | 13/0 |
| P value | n.s. | n.s. | 0.05 |

1 Mean of 14 experiments. 2 Mean of 10 experiments. 3 Granulocytes or monocytes and erythrocytes. 4 n.s. = not significant.

Fig 7. Dose–response curve of daunorubicin for rhodamine dull (A) and bright (B) CD34+ cells without and with 10 μM verapamil (corrected for the mean intrinsic toxicity of verapamil <10%). A significant (P<0.04) modulation of toxicity was measured in the rhodamine dull, not in the rhodamine bright cells.
cells. These results are even more relevant since the concentrations of anthracyclines used in our assay were in the same range of in vivo achievable concentrations (Speth et al., 1988).

Using optimal concentrations, Pgp modulators enhanced the toxic effects of anthracyclines on the rhodamine dull progenitors. Haematological toxicity of modulators has been reported in clinical trials (Figueroedo et al., 1990; Schetihauer et al., 1993; Bartlett et al., 1994). Pgp modulation on haemopoietic progenitors and also pharmacokinetik changes of the cytotoxic drug due to Pgp modulation on liver or kidney have been suggested. Nakarai & Koizumi (1990) and Visani et al. (1993) showed that calcium antagonists increased toxicity to haemopoietic progenitors in vitro. Whether these observations were due to specific Pgp modulation was not proved. Aspecific intrinsic or intracellular effects such as redistribution of the cytostatic drug were not excluded. Therefore we studied the effects of verapamil on progenitors with a functional high and low or negligible Pgp. In accordance with the high Pgp expression, increased toxicity of anthracyclines in the presence of verapamil was significant only for the rhodamine dull cells. Thus the increased toxicity was the result of specific Pgp modulation of the haemopoietic progenitors. This clonogenic assay is an appropriate tool to test efficacy, aspecific (intrinsic) haematological toxicity and specific Pgp mediated toxicity of potential Pgp modulators in combination with anticancer drugs.

Chaudhary & Roninson (1991) showed an inverse correlation between rhodamine retention and clonogenic capacity in normal bone marrow cells. Other investigators confirmed the correlation between rhodamine retention and clonogenic capacity (Bertocellos et al., 1985; Mulder et al., 1987; Udomsaki et al., 1991). By means of a mouse transplant model it has been proved that the bone marrow repopulating capacity of cells can be predicted on the basis of rhodamine retention (Ploemacher et al., 1988). Within the CD34+ population, rhodamine retention was also inversely correlated with clonogenic capacity. The rhodamine dull cells showed a higher clonogenic and proliferative capacity expressed as a higher number, with larger and more mixed colonies. We confirmed the more primitive origin of progenitors with a high Pgp expression, the CD34+ rhodamine dull cells. The more primitive progenitors are protected against various toxic agents by this physiological defensive mechanism. We showed that this can be modulated very effectively by verapamil in this fraction, which is held responsible for haemopoietic recovery. After cytoreductive therapy in the presence of a Pgp modulator, the toxicity will increase most abundantly in the more primitive rhodamine dull progenitors. Morbidity and mortality due to this toxicity might counterbalance the improved anticancer efficacy. An approach for circumventing unacceptable haematological toxicity is reinfusion of bone marrow or peripheral stem cells. To make dose intensification feasible, up-regulation of MDR1 expression in haemopoietic stem cells by means of transfection of MDR1-cDNA with the use of retroviruses has also been suggested (Bertolino et al., 1994; Ward et al., 1994; Schwarzenberger et al., 1996). Our results prove the clinical significance of MDR1 function at least in the more primitive CD34+ rhodamine dull cells. Although by means of PCR or MoAbs MDR1 expression seems to be low, functionally it appeared important in protecting and reducing sensitivity to anticancer drugs such as anthracyclines. MDR-1 transfection is not likely to improve the resistance of primitive progenitor cells to Pgp-dependent drugs.

In this study we showed that the more primitive progenitor cells within the CD34- bone marrow fraction have a functionally important Pgp. We showed that this pump plays an important role in protecting these cells against pharmacological attainable anthracycline concentrations. Effective Pgp modulators will increase the toxicity within this progenitor fraction; held responsible for haemopoietic recovery. We have proved that the potentiation of anthracycline toxicity in these cells is the result of specific Pgp modulation on these cells.

REFERENCES


