Abstract. Background/Aim: P-glycoprotein (Pgp) expression in neoplastic cells is known to reduce cell sensitivity to several cytotoxic Pgp substrates. A member of the ABC transporter family, Pgp, represents the most frequently described membrane efflux pump and its expression in neoplastic cells is responsible for multi-drug resistance. Several lines of evidence indicate that the expression and increased function of both Pgp and glucosylceramide synthase (GCS, an enzyme responsible for ceramide pathway de-activation in the regulation of apoptosis progression) enhance the resistance of Pgp-positive cells. Previously, we described a reduction in the uridine diphosphate (UDP)-glucose contents of mouse leukemia cells (R) expressing Pgp due to vincristine selection compared to parental L1210 cells (S). The reduced availability of UDP-glucose as a glucose donor in R cell glycosylation reactions could limit GCS-catalyzed ceramide glycosylation. Consequently, over-expression of Pgp in Pgp-positive L1210 cells may be associated with reduced ceramide glycosylation. Materials and Methods: To test this idea, we measured the expression and activities of Pgp and GCS, UDP-glucose levels, cellular uptake of C12-NBD-ceramide (a fluorescent analogue of ceramide) and ceramide-induced cell death in S and R cells. T-cells, another Pgp-positive variant of L1210 cells that express Pgp due to their transfection with a gene encoding human Pgp were also used in this study. Results: We detected significantly reduced levels of C12-NBD-ceramide glycosylation and reduced UDP-glucose contents in Pgp-positive R and T-cells compared to S cells. C12-NBD-ceramide uptake assays revealed nearly identical dynamics of uptake time-dependency curves. The Pgp-positive L1210 variants (R and T) are more sensitive than Pgp-negative S cells to ceramide-induced cell damage, as measured by an fluorescein isothiocyanate-labeled annexin V and propidium iodide apoptosis necrosis kit. Short chain C2-ceramide was more effective at inducing cell damage than ceramide analogues with longer chains. Conclusion: These evidence indicates that the down-regulation of UDP-glucose contents in Pgp-positive L1210 cells is responsible for their collateral sensitivity to ceramide-induced apoptosis.

Over-expression of P-glycoprotein (Pgp), an ABCB1 member of the ABC transporter family, is the most frequently described molecular cause of neoplastic cell resistance to various cytotoxic Pgp substrates and the development of multidrug resistance (MDR) phenotypes associated with Pgp represents a real obstacle for effective chemotherapy of various neoplastic diseases (1, 2). Increased transcription of Pgp and glucosylceramide synthase (GCS) was observed when samples from patients with drug-resistant and drug-sensitive leukemias were compared (3). Ceramide and other sphingolipids act as second messengers and regulate the function of several target proteins including specific protein kinases, protein phosphatases and proteins involved in apoptosis entry and progression (4). The effectiveness of several anticancer agents could be addressed, at least partially, with regards to their ability to activate ceramide-mediated pathways in cancer cells (5). GCS is known to block the apoptotic effects of ceramides by coupling them to glucose, which is thought to de-activate them (6). Resistance to short-chain ceramide anti-proliferative effects was shown to depend on the transcriptional activity of both Pgp and GCS in cervical cancer cells (7). These facts indicated that Pgp-mediated drug resistance and ceramide regulatory pathways

Key Words: L1210 cells, P-glycoprotein, glucosylceramide synthase, UDP-glucose, ceramide induced cell death.
cooperate in the escape of programmed cell death (8). This cooperation could not be directly addressed to connect Pgp-mediated MDR and GCS-catalyzed ceramide glycosylation as GCS inhibition by specific inhibitors does not reverse the resistance (9).

Uridine diphosphate (UDP)-glucose is a glucose donor in the reaction catalyzed by GCS. Recently, we described reduced intracellular levels of UDP-saccharides, particularly UDP-glucose, when Pgp-positive mouse leukemia cells (R) were compared to Pgp-negative L1210 cells (S) (10). R cells were obtained by selecting S cells for resistance with vincristine (11). Reduced UDP-glucose levels in R cells could limit the net in situ ceramide glycosylation. To prove this idea, we compared S and R cells with regards to their Pgp and GCS expression and activity levels, intracellular UDP-glucose levels and ceramide-induced apoptotic effects. To determine whether changes in ceramide glycosylation in Pgp-positive L1210 cells are directly related to expression of Pgp we compared S and R cells with cells obtained by transfecting S cells with a gene encoding human Pgp (T-cells) (12).

Materials and Methods

Cell cultivation conditions. The following L1210 cell variants were used in this study: (i) S, drug-sensitive parental cells; (ii) R, Pgp-positive, drug-resistant cells over-expressing Pgp due to vincristine selection (Gedeon Richter Co., Budapest, Hungary) (11); and (iii) T, Pgp-positive, drug-resistant cells over-expressing Pgp due to stable transfection with the Pgp (12) from the Addgene plasmid 10957 (pHaMDRwt), a retroviral plasmid encoding full-length Pgp (13). The cells (S, R and T; 1×10⁶ cells) were cultured in 4 ml RPMI 1640 (pHaMDRwt), a retroviral plasmid encoding full-length Pgp (13). The cells were incubated for two passages without vincristine prior to each experiment.

Estimation of Pgp and GCS expression by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from S, R and T cells using TRIzol reagent (Life Technology, Bratislava, Slovakia) according to the manufacturer’s instructions. Reverse transcription was performed using 2 μg of DNAse I (Thermo Fisher Scientific, Bremen, Germany)-treated total RNA and the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. PCR was performed in a 25 μl total volume containing 4 μl RT mixture, 1× PCR buffer, 1.5 mmol/l MgCl₂, 0.2 mmol/l dNTP, 1 pmol of each gene-specific primer set and 0.3 U DyNazyme II DNA polymerase (Finnzymes OY, Espoo, Finland) in the buffer provided by the reverse transcriptase. The samples were subjected to 35 cycles of denaturation (95°C, 45 sec), annealing (57°C Mus musculus and Homo sapiens Pgp, 58°C GCS and 56.6°C GAPDH; 30 sec) and extension (72°C, 90 sec), followed by a final extension at 72°C for 10 min. The PCR products were separated on a 1.7% agarose gel (Invitrogen, Life Technology, Bratislava, Slovakia) and visualized with GelRed™ nucleic acid gel stain (Thermo Fisher Scientific) using a Typhoon 9210 Imaging System (GE Healthcare, Wauwatosa, WI, USA, formerly Amersham Biosciences).

The primer sets employed in this study were as follows: GAPDH, F: 5’-TGA ACG GGA AGC TCA CTG G-3’, R: 5’-TCC ACC ACC CTG TTG CTG TA-3’; Mus musculus Pgp, F: 5’-GGC TGT TAA AGG TAA TCA CTC-3’, R: 5’-TGT TCT CTT ATG AAT CAC GTA-3’; Homo sapiens Pgp, F: 5’-AGA CAT GAC GAT GTA TGC-3’, R: 5’-CTC CTG TCG CAT TAT AGC-3’; GCS, F: 5’-CGG TAT AGC AAG CTC CCT GG-3’, R: 5’-CAA GAC ATC CCC GTC ACA CA-3’.

Examination of Pgp function in S, R and T cells using the calcine/acetoxyethyl (AM) assay. Pgp transport activity was measured using a previously described calcine retention assay (14, 15). Cells were centrifuged (500 xg), washed three times with PBS containing 0.2% BSA and, re-suspended in 500 μl of the same buffer. Calcine/AM (final concentration 0.1 μmol/l, Sigma-Aldrich, St. Louis, MO, USA) was added directly to the buffer and the samples were incubated for 20 min at 37°C in a CO2 incubator. Verapamil (10 μmol/l) was used as a Pgp inhibitor and was added with Calcine/AM. Propidium iodide (final concentration 0.9 μmol/l, Sigma-Aldrich) was then incubated with the cells for an additional 10 min and, finally, the cells were washed twice with ice-cold PBS. Fluorescence was measured using an Accuri C6 flow cytometer (BD Bioscience, San Jose, CA, USA). Only viable, propidium iodide-negative cells (greater than 92% in each case) were counted.

Cellular ceramide glycosylation assay. S, R and T cells (10⁶) were preincubated for 120 min in cultivation medium in CO2 incubator with GCS inhibitor DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP, 0.1 and 1 μmol/l, stock solutions were prepared in dimethyl sulfoxide (DMSO) at final concentrations not exceeding 0.2%), Pgp inhibitors verapamil (10 μmol/l) and ketoconazole (10 μmol/l) or Pgp substrate vincristine (0.2 μmol/l). The control cells were processed in parallel in the absence of these substances. The cells were then transferred into 500 μl of 1% BSA RPMI medium containing the appropriate substances and 100 μmol/l nitrobenzoaxidazol (NBD)-C12-ceramide complexed with BSA and incubated for an additional 120 min. Following the incubation, the cells were washed two times with PBS (pH 7.4), scraped with 400 μl of ice-cold acidic methanol (acidic acid: methanol, 1:50, v/v). The lipids were extracted by the addition of chloroform (400 μl) and water (400 μl). After centrifugation (5 min, 10,000 rpm), the lower organic phase was collected into new tubes. The lipids were then re-extracted from the upper phase by the addition of chloroform (500 μl) and centrifugation (5 min, 10,000 rpm). The lower organic phases were pooled, vacuum evaporated and re-suspended in chloroform/methanol (100 μl, 1:1, v/v). Samples (30 μl) were applied to high performance thin layer chromatography (HPTLC) plates and developed with chloroform/methanol/20 % ammonia (70:30:6.25, v/v/v) mobile phase. The HPTLC plates were then analyzed using a Typhoon 9210 phosphoimager (Amersham Biosciences, Piscataway, NJ, USA).

Estimation of cellular UDP-glucose contents. The levels of UDP-glucose were estimated as previously described (16). Briefly, cells were pelleted by centrifugation (3 min, 1,200 xg) and washed in ice-cold PBS. The pellets were twice extracted by 0.5 ml of 60% ethanol at 60°C for 15 min. The extracts were cleared by centrifugation and the supernatants were dried in vacuum drier and dissolved in 50 μl of 0.1 mol/l Tris-HCl buffer pH=8.5. The resulting solutions were used for spectrophotometric estimation of UDP-glucose contents using 0.02 units NAD/UDP-glucose dehydrogenase (Sigma-Aldrich) in 200 μl of reaction medium with 1.5 mmol/l NAD. The absorbances were read at 340 nm and
monitored over time intervals of 0-60 min. The quantities of UDP/glucose were calculated from the absorbance differences and a standard curve was obtained using UDP-glucose solutions.

Measurement of cell death induced by C2-, C6- and C8-ceramides. Cells (10^6 cells per ml) were incubated for 120 min with or without C2-, C6- or C8-ceramides (3 and 5 μmol/l, stock solutions were prepared in DMSO with final concentrations never exceeding 0.2 %) under standard culture conditions. The proportions of apoptotic and necrotic cells were then detected using a fluorescein isothiocyanate-labeled annexin V (FA V) and propidium iodide (PI) kit (Calbiochem, San Diego, CA, USA). According to the procedure described by the manufacturer, the cells were washed twice with PBS and gently resuspended in binding buffer (obtained from the manufacturer) containing 0.5 μg/ml FAV. The mixture was then incubated in the dark for 15 min at room temperature and centrifuged (2,500 rpm, 15 min). The resulting sediments were re-suspended in binding buffer, propidium iodide (final concentration 0.6 μg/ml) was added to each sample and the samples were analyzed by flow cytometry using an Accuri C6 flow cytometer (BD Bioscience).

Results

Pgp expression and activity in S, R and T cells. Both R and T-cells expressed massive quantities of the Pgp mRNA (Figure 1A); in contrast, Pgp expression in S cells was much less pronounced and only weak, if any, signal could be detected by RT-PCR.

Calcine retention assay was applied for detection of Pgp transport activity in S, R and T cells. Substantial calcine retention was observed for parental S cells (Figure 1B), while reduced calcine retention was observed for R and T-cells. Treatment with verapamil (17) restored the extent of calcine retention to that observed for S cells. Cyclosporine A (14) and ketoconazole (18), other inhibitors of Pgp, also restored calcine retention in R and T cells (data not shown).

GCS expression and cellular glycosylation of C12-NBD-ceramide. Nearly equivalent quantities of GCS transcripts
were detected in S, R and T-cells (Figure 1A). Cellular GCS production of glycosylated ceramides was detected by measuring glycosylation of C12-NBD-ceramide (19), a fluorescent analog of long chain ceramides, in extracts from C12-NBD-ceramide-loaded cells using HPTLC chromatography (Figure 1C). S, R and T-cell C12-NBD-ceramide loading led to similar extents of cellular retention of this substance. In contrast with the lactosylated and galactosylated C12-NBD-ceramides that were undetectable in cell extracts from all three cell types, measurable amounts of C12-NBD-glucosyl-ceramide were detected. A much greater quantity of this substance was detected in S cells than in either R or T cells indicating that GCS catalyzed conversion of ceramides into glucosyl-ceramides more effectively in S cells than in either R or T cells (Figure 1C), regardless of the nearly equivalent GCS expression levels (Figure 1A). It should be stressed-out that Pgp over-expression is associated with a down-regulation in ceramide glycosylation in both Pgp-positive variants of L1210 cells.

PPMP, a known inhibitor of GCS (20), inhibited GCS activity in S, R and T cells at a concentration of 1 μmol/l, while a 10-fold lower concentration was ineffective (Figure 1D). Verapamil, a known Pgp inhibitor, induced an increase in GCS activity primarily in R and T-cells. In contrast, another Pgp inhibitor, ketoconazole, did not affect GCS activity in any L1210 cell variants. Similarly to ketoconazole, vincristine (also a Pgp substrate) did not affect GCS activity in S, R or T cells (Figure 1D).

**Time-dependence of S, R and T cell C12-NBD-ceramide uptake and UDP-glucose levels.** To exclude the possibility that Pgp can eliminate this ceramide analogue from R and T cell intracellular space and, thus, limit ceramide glycosylation, we must compare the time-dependence of C12-NBD-ceramide uptake. Figure 2A shows the flow cytometry histograms of C12-NBD-ceramide uptake for S, R and T-cells during 0.5-120 min intervals. These data indicate similar dynamics for S, R and T cell C12-NBD-ceramide loading. The time-dependence measured for R and T-cells (Figure 2B) revealed only minor and negligible reduction in C12-NBD-ceramide retention compared to S cells. This small change in C12-NBD-ceramide uptake cannot be responsible for the differences in C12-NBD-ceramide glycosylation that are documented in Figure 1C.

Parental Pgp-negative S cells contained 2.84±0.15 nmol UDP-glucose per mg of cellular protein (Figure 3), while both R and T cells contained less than half of that detected in S cells. Induction of cell death in S, R and T cells by C2-, C6- and C8-ceramides. Incubation of cells in the absence or presence of 3 or 5 μmol/l C2-ceramide for 120 min (Figure 4) induced cell death effects and the majority of damaged cells were in
late apoptosis as they were stained by both FA V and PI. Following this incubation, more pronounced cell death effects were observed for R and T cells than in S cells as 10-15% of R and T cells were stained with both FA V and PI compared with 5-6% in S cells. A substantial fraction of cells were viable after this treatment (i.e., stained by neither FA V nor PI): approximately 85 % of S cells and 75 % of R and T-cells. C2-ceramide-induced cell death effects were monitored using FACS dot plots (Figure 4) and appeared to be related to apoptosis, with little contribution from necrosis.

Similar procedures were also used to monitor the cell death effects of 3 μmol/l C6- and C8- ceramides; the effects of these ceramides are summarized in Table I. In S cells, only C2-ceramide showed significant effects on cell death. C2-ceramide was also the most effective against R and T cells, although significant cell death effects were also observed for R cells with C6- and C8-ceramides. If the latter two ceramides also induced cell death effects in T-cells, the effects were less pronounced and the results indicate only marginal significance.

Discussion

Massive contents of Pgp mRNA in R and T-cells when compared to S cells are visible on Figure 1A. We routinely verified the Pgp-positive status of R and T cells and Pgp-negative status of S cells by Western blotting and immunocytochemistry using an anti-Pgp antibody documented in our previous papers (15, 21, 22).
Cells were incubated for 2 h in growth medium with or without 3 μmol/l C8 ceramide 93.7±2.1 2.6±0.5 1.1±0.2 2.6±0.4
C6 ceramide 90.7±2.0 2.5±0.2 2.9±0.3 * 3.8±0.3*
C8 ceramide 88.3±1.9 * 4.0±0.4* 2.6±0.5 5.1±0.7
C6 ceramide 84.3±2.1 ** 1.9±0.3 8.6±0.5 ** 5.2±0.3*
C2 ceramide 75.7±2.3** 7.3±0.3** 4.1±0.3* 12.9±0.5 ** 5.1±0.7
Control 93.2±1.8 2.2±0.5 1.5±0.4 3.1±0.4

Cells were incubated for 2 h in growth medium with or without 3 μmol/l C2-, C6 or C8 ceramides. The progression of apoptosis and necrosis was assayed using a fluorescein isothiocyanate-labeled annexin V (FA V) and propidium iodide (PI) kit. The fluorescence of specific cells was assayed using fluorescence flow cytometry. Data are expressed as the proportion of stained cells in respective dot blot quadrants (see figure 4) and represent the mean±S.E.M. of three independent values. Unstained cells- measured using fluorescence flow cytometry. Data are expressed as the proportion of cells in respective dot blot quadrants (see figure 4) and represent the mean±S.E.M. of three independent values. Unstained cells-

When esterified, calcein/AM acts as an intracellular calcium indicator and is a suitable Pgp substrate (23). Active Pgp blocks calcein/AM entry into Pgp-positive cells. After intracellular deesterification, liberated calcein is not a Pgp substrate and is capable of binding Ca2+ to produce a fluorescent complex (24); therefore, Pgp efflux activity blocks calcein activation and fluorescence in Pgp-positive cells (15). Reduced calcein retention has frequently been used as a measure of efflux activity at the plasma membrane of MDR cells (25, 26) and may be antagonized by Pgp inhibitors, such as verapamil (14, 17). Thus, the depressed retention of calcein documented in Figure 1B for the R and T cells compared to the S cells represents a measurable marker of Pgp efflux activity in these cells. Both R and T cells represent Pgp positive MDR cell variants with reduced sensitivity to Pgp substrates (12).

In our experiments, down-regulation of cellular glycosylation of C12-NBD-ceramide was observed in both Pgp-positive cell variants (R and T) when compared with S cells (Figure 1C). In contrast with these observations, up-regulation of ceramide glycosylation was observed in MCF7 cells over-expressing Pgp that had been selected for adriamycin-resistance during treatment with these inhibitors (27). This discrepancy indicates that crosstalk between Pgp-mediated MDR and ceramide metabolism pathways may have different effects in different cells depending on their molecular characteristics. If Pgp protects R and T-cells from C12-NBD-ceramide uptake, C12-NBD-ceramide cannot be glycosylated by GCS. Contrary to this idea, S, R and T cells loaded with C12-NBD-ceramide had nearly equivalent amounts of this substance (Figure 1C). Pgp was described for its involvement in flipping several NBD-labeled simple glycosphingolipids, including NBD-glucosylceramides, from one leaflet of the bilayer to the other in an ATP-dependent and vanadate-sensitive manner (28). Unfortunately, the authors failed to estimate the leaflet distribution of C12-NBD-ceramide as NBD-lipids lacking a hydrophilic head group, such as glucose that traffic between lipid bilayer leaflets too rapidly. To exclude the possibility that Pgp can eliminate this ceramide analogue from R and T-cell intracellular space and, thus, limit ceramide glycosylation, we measured the dynamics of C12-NBD-ceramide retention within S, R and T-cells. However, only slight differences between S, R and T cells in C12-NBD-ceramide retention were observed (Figure 2). These differences could not be responsible for depression of cellular glycosylation of C12-NBD-ceramide in R and T-cells when compared to S cells.

Both Pgp-positive cell variants R and T differ from S cells in decreased levels of UDP-glucose cellular contents (Figure 3). Strong reductions in R cell UDP-glucose compared with S cells were detected previously (10). Interestingly, nearly identical reductions in UDP-glucose levels were observed for T and R cells. Consequently, Pgp expression, regardless of the manner of expression, is responsible for reductions in UDP-glucose levels in L1210 cells. Decreases in UDP-sugar pools influence the transglycosylation reactions that alter the intracellular glycogen content, cell surface acidic sugar levels, the intracellular glycoprotein profile (10) and ceramide glycosylation (Figure 1C and B). These changes are also involved in the extensive remodeling of cell surface saccharides of Pgp-positive R and T cells, compared with S cells, that can be monitored using various lectins (12, 29, 30) and may be involved in the reduced sensitivity of R and T cells to the protein N-glycosylation inhibitor tunicamycin (15).

Sorting of apoptotic and necrotic cells after staining with FA V and PI can be used to estimate the effects of ceramide-induced damage (31). Data shown in Figure 4 and Table I demonstrate the increased sensitivity of Pgp-positive R and T-cells to ceramides (particularly C2-ceramide) over that of S cells. Furthermore, R cells appear to be more sensitive to ceramides than T-cells. In R and T cells, Pgp over-expression is associated with collateral sensitivity to membrane permeable ceramides. Considering these data, our Pgp-
positive leukemia cells differ from others in which Pgp over-expression is associated with resistance to short-chain ceramide antiproliferative effects (7).

The present study suggests that Pgp over-expression in R and T-cells is associated with a decrease in UDP-glucose cell contents when compared with Pgp-negative S cells. This occurred regardless of the source of Pgp expression, either by vincristine-resistance selection or transfection with a gene encoding human Pgp. Lower levels of UDP-glucose are responsible for the reduction in cellular ceramide glycosylation that confers enhanced sensitivity to ceramides, particularly those with short chains. In this regard, R and T cells differ from other MDR cancer cell models in which the MDR phenotype is associated with an increased capacity for ceramide glycosylation that prevents ceramide-induced apoptosis (32).

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