European Journal of Pharmacology 609 (2009) 34–39

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Glucosylceramide synthase inhibitors sensitise CLL cells to cytotoxic agents without reversing P-gp functional activity

Gareth Gerrard [a,1], Terry D. Butters [b], Kanagasabai Ganeshaguru [a], Atul B. Mehta [a,*]

[a] Department of Haematology, University College London Medical School, Royal Free Campus, London, UK
[b] Oxford Glycobiology Institute, University of Oxford, Oxford, UK

ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form 24 February 2009
Accepted 3 March 2009
Available online 12 March 2009

Keywords:
MDR
P-glycoprotein
CLL
Gaucher Disease
Glucosylceramide synthase inhibitor

ABSTRACT

Malignant B-cells from most chronic lymphocytic leukaemia (CLL) patients over-express MDR1 encoded P-glycoprotein (P-gp) multidrug efflux pump. Inhibition of glucosylceramide (GC) synthesis has been shown in cell lines to correlate with the expression and function of P-gp and sensitise cancer cells to cytotoxic agents. We investigated the hypothesis that reducing intracellular GC levels will reduce P-gp expression in malignant cells from CLL patients. We studied the ability of glucosylceramide synthase (GCS) inhibitors N-butyl-deoxygalactonojirimycin (OGB-1) and N-nonyl-deoxygalactonojirimycin (OGB-2) to sensitise CLL cells to conventional cytotoxic drug 2-chlorodeoxyadenosine (CdA) and the cytostatic drugs chlorambucil and fludarabine. The effect on P-gp activity was analysed using the calcein-AM accumulation assay where a multidrug activity factor (MAF) of >10 in the presence of a P-gp inhibitor denotes P-gp functional activity. The P-gp over-expressing cell line CEM-VLB showed a MAF value of 96.4 with the P-gp inhibitor Z.3HCL, which fell to 15.7 after co-incubation with OGB-1 and 45.9 with OGB-2. The IC_{50} for vincristine fell from >10 μg/ml to 55.5 ng/ml in the presence of OGB-2. In $P\text{-gp}^{+ve}$ peripheral blood mononuclear cells from three normal volunteers, the mean MAF values for Z.3HCL, OGB-1 and OGB-2 were 23.86, 1.83 and 16.2 respectively. In 9/13 CLL samples the mean P-gp functional activity was 22.15 and P-gp was over-expressed in 12/13 samples. However, the MAF value with OGB-1 and OGB-2 was <10. Nevertheless, sensitisation in CLL cells was observed by a reduction in the IC_{50} in the presence of OGB-1 and OGB-2 with the conventional drugs. We conclude that although GCS inhibitors sensitize CLL cells to cytotoxic and cytostatic drugs, they do not appear to have any effect on P-gp functional activity.

1. Introduction

A significant factor that contributes toward failure of treatment with cytotoxic and cytostatic agents in haematological malignancies is the over-expression of efflux membrane proteins resulting in multidrug resistance (MDR)(Gottesman et al., 2002; Leonard et al., 2003). The first described and the most extensively studied MDR efflux protein is the MDR1 encoded 170 kDa P-glycoprotein (P-gp) that results in resistance to many structurally unrelated drugs (Juliano and Ling, 1976). Studies in various haematological malignancies have shown over-expression of P-gp in a proportion of patients either at presentation or at relapse (Leith et al., 1999). In acute myeloid leukaemia (AML), good correlation between over-expression of P-gp, response to treatment and overall survival has been reported (Hunault et al., 1997). A mechanism of circumvention of MDR is the use of non-toxic modulators or chemo-sensitizers that act competitively or inhibit P-gp activity. The well known modulators are verapamil (Yusa and Tsuruo, 1989), cyclosporine A (List et al., 2001) and its derivative PSC833 (Advani et al., 1999) and quinidine (Wishart et al., 1994), all acting as substrates of P-gp. Zosuquidar hydrochloride (Z.3HCL) is a potent and selective modulator that functions by inhibiting P-gp activity and thus drug efflux (Gerrard et al., 2004; Green et al., 2001; Dantzig et al., 1996).

B-chronic lymphocytic leukaemia (CLL) is characterised by the accumulation of monoclonal B-lymphocytes in the peripheral blood, bone marrow and the lymph node (Matutes et al., 1994). The clinical course is highly variable, ranging from rapidly advancing to clinically stable disease (Wagner and Cwynarski, 2004). When treatment is required, initial response may be followed by chemotherapy resistance and disease progression (Oscier et al., 2004). Cells from most patients with CLL intrinsically over-express P-gp, although its significance to clinical drug resistance of this disease is unclear (Consoli et al., 2002; Jamroziak et al., 2004). Many of the active agents used in the treatment of CLL (e.g. chlorambucil, purine analogues) are not P-gp substrates.

The basic physiological function of P-gp centres on protecting the cell from exogenous toxins. In addition to its action as an efflux pump, P-gp has the capacity to translocate short chain lipid molecules (including spingomyelin and glucocylceramide) across the plasma

* Corresponding author. Department of Haematology, Royal Free Hospital, Pond Street, London NW3 2QG, UK.
E-mail address: atul.mehta@royalfree.nhs.uk (A.B. Mehta).
[1] Current address: Department of Haematology, Imperial College London, Hammersmith Hospital, London, UK.

0014-2999/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2009.03.018

membrane (van Helvoort et al., 1996). Elevated levels of glycolipids have been shown to correlate with MDR in tumour cell lines (Lavie et al., 1996) and in primary tumour cells (Lucci et al., 1998). Cells modified by the introduction and consequent expression of high levels of glucosylceramide synthase (UDP-glucose-ceramide glucosyltransferase, EC 2.4.1.80)(GCS) showed resistance to various cytostatic drugs compared to unmodified wild type cells (Liu et al., 1999). The MDR modulating agents verapamil and cyclosporine A block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells (Lavie et al., 1997). Use of the PDMP (D-threo-1-phenyl-2-decanonylamino-3-morpholino-1-propanol) family of GCS inhibitors can reverse the MDR phenotype of MDR expressing cell lines (Olshefski and Ladisch, 2001). Genetically-induced over-expression of another MDR protein, multidrug resistance protein (MRP1) was accompanied by enhanced levels of glucosylceramide (Kok et al., 2000). Others have reported that inhibition of GCS does not sensitise tumour cells for anticancer drugs (Veldman et al., 2003) and the over-expression of GCS does not protect cells from drug induced detrimental effects of anticancer drugs (Tepper et al., 2000).

The n-alkylated imino sugars, N-butyl-deoxygalactonojirimycin (OGB-1) and N-nonyl-deoxygalactonojirimycin (OGB-2) are selective inhibitors of GCS (Platt et al., 1994a,b; Andersson et al., 2000) (Fig. 1A). These small molecule compounds are currently undergoing *in vitro* and *in vivo* evaluation as substrate reduction therapy for the lysosomal storage disorder Gaucher disease. However, as GCS inhibitors, they may have an impact on P-gp as observed with the PDMP family of GCS inhibitors (Lavie et al., 1997; Olshefski and Ladisch, 2001). Structural similarities between GCS inhibitors and glycolipids may make them substrates for P-gp (Butters et al., 2003). Gaucher disease is the most common lysosomal storage disease, characterised by a deficiency of the enzyme glucocerebrosidase, which leads to an accumulation of its substrate glucocerebroside (Cox et al., 2003; Elstein et al., 2004). Gaucher disease is associated with an increased incidence of B-cell malignancies (e.g. non-Hodgkin lymphoma, CLL and myeloma); and drug resistant acute myeloid leukaemia has also been reported (Bohm et al., 2001; Krishnan et al., 2003).

Consequently, we hypothesised that inhibition of GCS may reverse P-gp efflux and thus overcome MDR (Fig. 1B). Since malignant cells from CLL patients intrinsically over-express P-gp, we used them as an appropriate model to review the role of GCS activity. We analysed the effect of the GCS inhibitors OGB-1 and OGB-2 on P-gp functional activity and used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colourimetric assay to assess their ability to sensitise CLL cells to the cytotoxic and cytostatic effect of conventional agents used in their treatment i.e., 2-chlorodeoxyadenosine (CdA), chlorambucil and fludarabine. Z.3HCL was used as a control inhibitor of P-gp function.

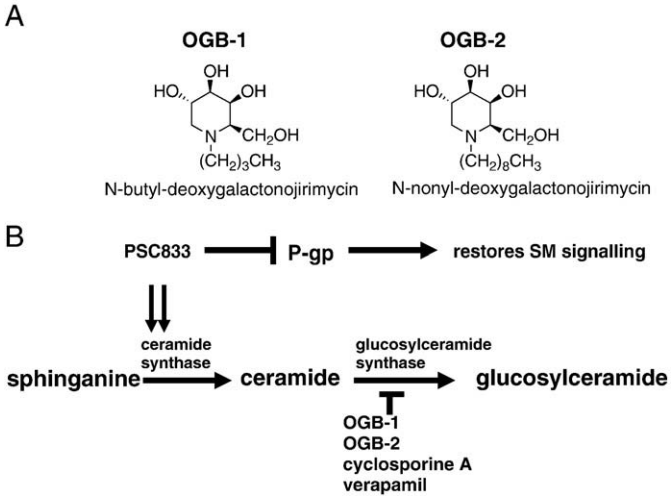

Fig. 1. A: The chemical structures of OGB-1 and OGB-2. B: The effect of P-gp inhibitors in glycolipid metabolism. PSC 833, cyclosporine A, verapamil are known inhibitors of P-gp activity. ⊣, ⊤ inhibition, ↓↓ enhancement.

Table 1
Clinical, demographic, and P-gp status of CLL patients.

Patient	Sex	Age: years	WBC 10^9/l	Lymp 10^9/l	P-gp ratio	Treatment
1	M	67	71.6	58.2	nd	Rx
2	M	82	99.5	81.1	nd	Rx
3	M	82	41.5	31.8	1.6	unRx
4	F	77	52.4	38.5	1.7	Rx
5	M	83	53.6	43.9	1.6	unRx
6	M	62	42.0	nd	1.5	Rx
7	M	69	69.9	58.5	2.8	Rx
8	M	79	19.3	nd	1.7	unRx
9	M	70	14.6	10.6	2.9	unRx
10	F	73	50.0	nd	nd	unRx
11	M	63	19.0	10.4	2.5	Rx
12	F	63	15.5	10.6	1.1	Rx
13	F	82	32.1	24	nd	Rx
14	M	74	36.2	27.0	2.13	unRx
15	M	90	65.7	53.0	2.3	unRx
16	F	80	53.6	43.5	1.3	Rx
17	M	79	19.3	15.5	1.7	Rx

M = male; F = female; WBC = white blood cell count; Lymp = lymphocyte count; Rx = treated; unRx = untreated; nd = data unavailable.

2. Materials and methods

The study was approved by the ethics committee of the Royal Free Hampstead NHS Trust. Peripheral blood samples in preservative free heparin were obtained after informed consent from 3 normal volunteers and 17 patients with CLL. Clinical data are shown in Table 1. There were 12 males and 5 females with a median age of 77 years (range 62–90). The diagnosis of CLL was made on standard morphologic and immunophenotypic criteria, including raised peripheral blood lymphocyte count ($>5.0\times10^9$/l) with abnormal co-expression of CD5, CD19, weak expression of CD22, CD23, surface immunoglobulin light chain restriction and absent FMC7 expression. The white cell count ranged from 14.6–99.5 $\times 10^9$/l (median 42×10^9/l) and the lymphocyte count from 10.4–81.1 $\times 10^9$/l (median 35.2×10^9/l). Ten patients had been treated. All analysis was done on fresh samples within 24 h.

2.1. Separation of normal MNC and CLL cells

Samples were analysed by flow cytometry for T-cell contamination by labelling 100 µl whole blood with 10 µl of anti-CD2-FITC (BD Bioscience, UK) for 20 min and then treated with red cell lysing solution (in house) for 10 min prior to analysis. By gating the FL-1 CD2 channel, the proportion of $CD2^+$ T-cells to $CD2^-$ B-cells could be elucidated. The mononuclear cell fraction was then obtained from the whole blood samples by Lymphoprep (Nygaard, Norway) density gradient centrifugation. Samples with <90% B-cell purity were treated overnight with sheep red blood cell (TCS Biologicals, UK) rosetting to remove excess T-cells. The mononuclear layer was removed and washed twice in HBSS, and resuspended in 10 ml RPMI (Roswell Park Memorial Institute medium) + 10% foetal calf serum.

2.2. Maintenance of cell lines

Two cell lines were utilised: CCRF-CEM (CEM) and its P-gp over-expressing derivative CCRF-CEM/VLB_{100} (VLB) (Beck and Cirtain, 1982). The CEM (P-gp negative) line was cultured in RPMI + 10% foetal calf serum, with no additional antibiotics. The VLB line was cultured in the same medium but with the addition of 10 µM vinblastine (Sigma-Aldrich, UK) to maintain the P-gp phenotype. A double stock of VLB

cell cultures was kept in rotation with one flask containing vinblastine-free medium for use in experiments.

2.3. P-gp protein expression

P-gp expression was measured by flow cytometry using the MRK_{16} monoclonal antibody (TCS Biologicals, UK). The results are expressed as a ratio of median channel fluorescence (MCF) of the specific antibody relative to the MCF of isotype matched control serum. A MCF ratio of >1.1 was considered P-gp positive for over-expression (Gerrard et al., 2004).

2.4. P-gp functional assay

The P-gp functional assay was essentially similar to that described previously (Gerrard et al., 2004; Karaszi et al., 2001). Briefly, cells at 0.5×10^6/ml were incubated for 24 h at 37 °C with either 500 μM OGB-1, or 10 μM OGB-2 along with a control. The concentration at which each OGB compound used was derived from earlier cell toxicity studies (data not shown). At 24 h, 1 ml aliquots of the cells were removed to 4 ml FACS tubes and half of the control cells were incubated with 100 nM Z.3HCL (Eli-Lily, UK) at 37 °C for 10 min. Calcein-AM (Sigma-Aldrich, UK) at concentration of 50 nM was then added to all the tubes and incubated at 37 °C for 10 min. The cells were then washed twice in PBS and resuspended in 150 μl PBS for analysis on the FACScan. P-gp functional inhibition was measured relative to the control cells as an increase in fluorescent intensity and expressed as MAF (multidrug activity factor) using the formula:

$$MAF = ((MCF_m - MCF_c)/MCF_m) \times 100$$

where MCF_m is the median channel fluorescence of the inhibited cells, and MCF_c is the median channel fluorescence of the unmodulated control cells.

2.5. MTT assay

The MTT assay was implemented as previously described (Ganeshaguru et al., 2002). Essentially cells were aliquoted into 96 well plates and incubated at 37 °C in the presence and absence of the GCS inhibitors OGB-1 and OGB-2. After 24 h, the cytostatic drugs CdA, chlorambucil and fludarabine (all from Sigma-Aldrich) for CLL cells or vincristine (VCR) for VLB cell line were added at $\log_{10}$ serial dilution concentrations in triplicates. At 72 h, 10 μl of MTT (5 mg/ml, Sigma-Aldrich) was added for an additional 4 h. The precipitated purple MTT formazan was centrifuged for 5 min at 600 *g*. The supernatant was removed and the formazan pellet was dissolved in 100 μl DMSO (dimethyl sulfoxide, Sigma-Aldrich)). The amount of purple formazan produced by the process is linearly proportional to the number of viable cells when read on a suitable plate reader (Anthos Labtec Instruments, Austria) and IC_{50} calculated using GraphPad Prism 4 software.

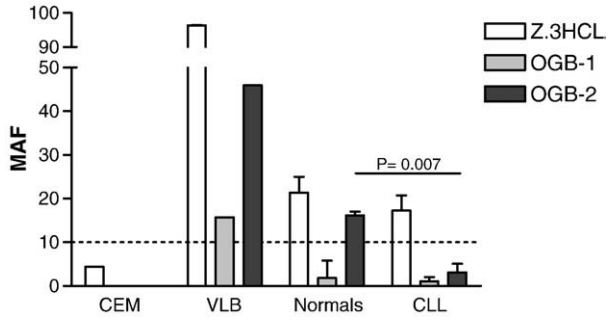

Fig. 2. P-gp calcein-AM functional assay and results expressed as MAF units. The horizontal broken line indicates the cut-off point for +ve P-gp activity. Results of *t*-tests performed between the matching Normal and CLL groups indicate a significant difference only for the OGB-2 group. CEM = P-gp −ve cell line; VLB = P-gp +ve cell line; normal = MNC from normal volunteers; CLL = cells from patients with CLL.

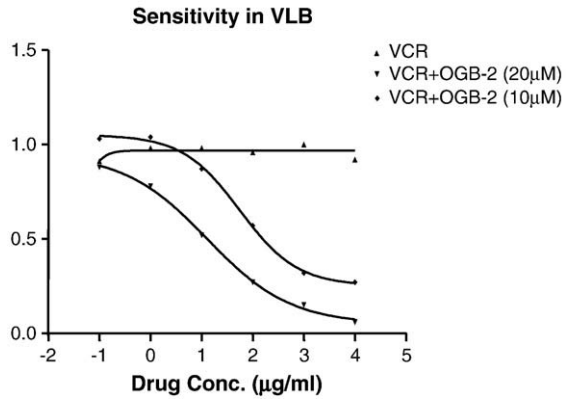

Fig. 3. Sensitivity of P-gp +ve VLB cell line to the presence of vincristine (VCR) alone and vincristine + OGB-2 at 10 μM and 20 μM. The vertical axis is a ratio of viable cells with increasing concentration of vincristine in relation to untreated controls at 72 h.

3. Results

The IC_{50} for OGB-1 and OGB-2 in CLL cells was >2 mM and 60 μM respectively (data not shown). Consequently, the non cytotoxic concentrations of 500 μM for OGB-1 and 10 μM for OGB-2 were used for P-gp functional and sensitisation studies.

3.1. P-gp expression (Table 1)

Thirteen of the patients with CLL were analysed for P-gp expression and 12 over-expressed P-gp (MCF ratio >1.1) with a median of 1.7, range 1.3–2.9 (Table 1).

3.2. P-gp function (Fig. 2)

The results of the calcein-AM P-gp functional inhibition assay in CLL cells is shown in Fig. 2. A MAF >10 represents a significant inhibition of P-gp efflux function. The CEM cell line, which is P-gp null, correspondingly showed a very low MAF for the potent P-gp inhibitor Z.3HCL, as well as for OGB-1 and OGB-2, where the MAF was 0. The VLB cell line showed a very high MAF for Z.3HCL, denoting both its strong P-gp expression and the potent ability of Z.3HCL to block calcein-AM export. OGB-1 and OGB-2 were also able to significantly inhibit P-gp function in the VLB cell line, with the latter being

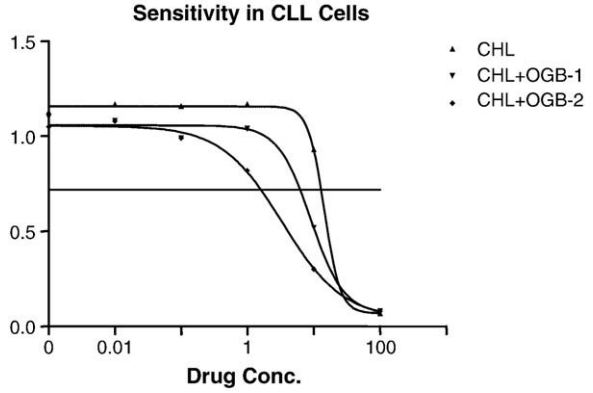

Fig. 4. A representative presentation of the sensitivity of CLL cells to chlorambucil alone or chlorambucil + OGB-1 or chlorambucil + OGB-2. The vertical axis is a ratio of viable cells with increasing concentration of either OGB-1 or OGB-2 in relation to untreated controls at 72 h.

significantly more potent. The normal controls were all shown to be expressing significant levels of P-gp, and this is correspondingly demonstrated in the calcein-AM results with both Z.3HCL and OGB-2 obtaining significant MAFs. OGB-1 did not show significant reduction. The CLL cells were also shown to over-express P-gp, and Z.3HCL shows a significant MAF. However, neither OGB-1 nor OGB-2 showed significant inhibition. *t*-test performed between the matching Normal and CLL inhibitor groups show no significant difference in inhibition for Z.3HCL and OGB-1, but indicate that P-gp efflux is significantly more inhibited by OGB-2 in the normals than in the CLL patients ($P = 0.007$).

3.3. MTT (Figs. 3–5)

The results of the MTT assay for the P-gp over-expressing VLB cell line and OGB-2 in combination with vincristine is shown in Fig. 3. The cytostatic drug and avid P-gp substrate vincristine was shown to be ineffective in killing VLB cells, with an inferred IC_{50} of > 10 μg/ml. The addition of 10 and 20 μM of OGB-2 overcame the resistance to vincristine and resulted in much reduced IC_{50}s of 0.056 and 0.013 μg/ml respectively.

The results of the MTT assay for CLL cells from patient 6 with and without chlorambucil and OGB-1 or OGB-2 is shown in Fig. 4. Chlorambucil alone resulted in an IC_{50} of 14.46 μg/ml. When in combination with OGB-1, this was reduced to 9.09 μg/ml, and when in combination with OGB-2 it was further reduced to 3.35 μg/ml. This combination is significant in its difference from the previous result, shown in Fig. 3, in that chlorambucil is not a P-gp substrate.

The results of MTT assays performed on CLL cells with the drugs CdA, chlorambucil and fludarabine with and without OGB-1 and OGB-2 are shown in Fig. 5A and B respectively. In a proportion of the samples, there was a significant decrease in the required concentration of the respective cytotoxic agent to achieve a 50% kill of the malignant cells compared to cells not pre-incubated with either OGB-1 or OGB-2. The graphs in Fig. 5 represent the mean IC_{50} derived from the mono and co-incubation of the patient cells with the cytostatic drugs and the OGB compounds. Paired *t*-tests were performed to assess the significance of any sensitisation; there was a significant sensitisation by OGB-1 and OGB-2 when co-incubated with CdA ($P = 0.0260$ and $P = 0.0105$, respectively) and fludarabine ($P = 0.0378$ and $P = 0.0107$, respectively), but not for chlorambucil (not significant for both OGB compounds). The degree of sensitisation (induced by the co-incubation) was calculated for each patient sample and from these the mean percentage sensitisation was derived and is displayed underneath the main body of the graphs. The values given appear slightly discordant as represented by the histograms, which represents the overall mean changes. However, the paired *t*-test performed for statistical analysis does take patient result pairing into account. The 50.4% reduction for CHL + OGB-1, although large, was not found to be significant probably because of the wide spread of data in a relatively small sample size.

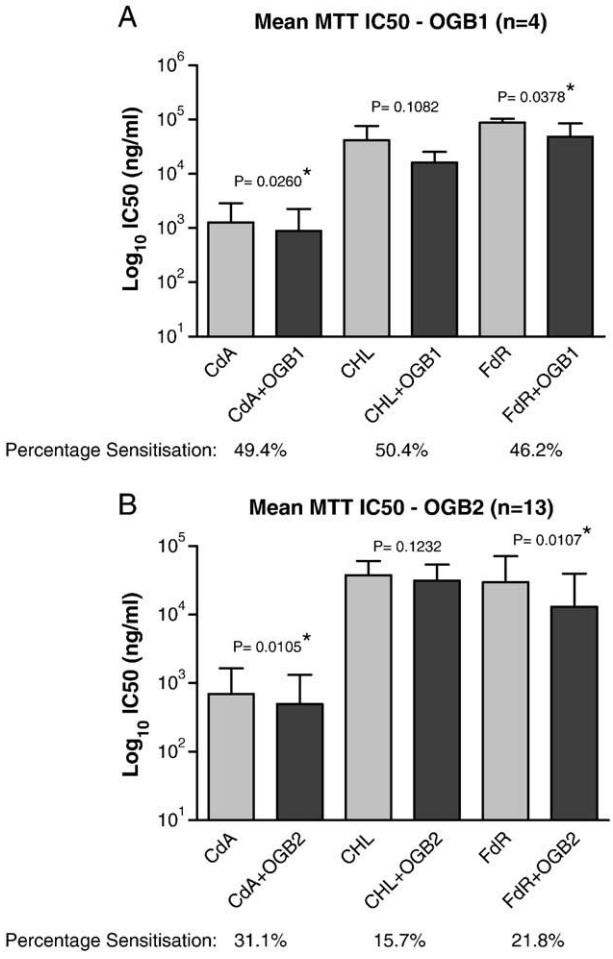

Fig. 5. Mean IC_{50} from MTT assays showing the level of sensitisation of CLL cells by OGB-1 and OGB-2 to the drugs CdA, chlorambucil (CHL) and fludarabine (FdR), compared to cells not pre-incubated with GCS inhibitors. One-tailed paired *t*-tests were performed to assess significance. The error bars denote standard deviation. Also shown is the mean percentage change in sensitivity induced by the addition of OGB. A in the presence of OGB-1. B in the presence of OGB-2.

4. Discussion

Glycosphingolipids play an important role in mediating drug resistance. Over-expression of the membrane efflux transporter P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter protein frequently underlies cancer cell resistance, but may also be important in microbial resistance (Gouaze-Andersson and Cabot, 2006). Furthermore, the essential role played by lipids, including glucosylceramide, lactosyl ceramide and gangliosides in cell development, apoptosis and pathogen/host interactions means that pathologic conditions characterised by disturbed lipid metabolism will display a diverse phenotype. Thus, although substrate accumulation within the reticuloendothelial system is the major clinical manifestation of Gaucher disease (glucocerebrosidase deficiency), there is a well recognised increased incidence of B lymphocyte malignancies, especially CLL and myeloma, and of AML (Krishnan et al., 2003). Pharmacologic inhibition of lipid synthesis by GCS inhibitors has been advocated not only as a therapeutic strategy in lysosomal storage disorders but also as chemotherapy (Cox et al., 2000; Radin, 1999).

Our study confirms that patients with CLL frequently over-express P-gp (Consoli et al., 2002; Jamroziak et al., 2004). This over-expression of P-gp was most likely intrinsic rather than acquired as there were almost equivalent numbers of treated and untreated patients in this study. Consequently we regarded cells from CLL patients to be an appropriate model to review the role of GCS activity in relation to MDR in general and P-gp more specifically in primary cells.

Nearly all previous studies on the interactions of P-gp and GCS in malignancy have been in cell lines, which have induced enhanced activity disproportionately higher than that seen in primary tumour cells. This may partly account for the disagreement in published studies on the role of GCS on MDR and P-gp. Many studies have shown significant correlation between elevated levels of GCS and MDR due to over-expression of P-gp (Lavie et al., 1996; Liu et al., 1999; Lucci et al., 1998). Other studies do not show similar correlation (Tepper et al., 2000; Veldman et al., 2003). Typically in AML cell line study, P-gp confers resistance to ceramide-induced apoptosis and inhibition of P-gp resulted in decreased GCS activity (Turzanski et al., 2005). However, in primary AML cells, GCS activity was raised in chemotherapy resistant compared to chemotherapy sensitive cells with no significant impact on P-gp expression (Itoh et al., 2003).

The N-alkylated imino sugars OGB-1 and OGB-2 have been shown to be selective inhibitors for GCS in studies aimed at substrate reduction therapy in Gaucher disease (Andersson et al., 2000; Platt et al., 1994a,b). In clinical trials, OGB-1 have also been shown to be well tolerated as oral agents with minimal adverse events (Cox et al., 2000; Elstein et al., 2004). These GCS inhibitors were therefore ideal candidates for studies to reverse MDR in malignant blood cells over-expressing P-gp. This study shows that CLL cells that over-express functional P-gp can be successfully modulated by the known P-gp inhibitor Z.3HCL. We have previously shown that Z.3HCL infusion can rapidly inhibit P-gp functional activity in P-gp over-expressing CD56+ cells (Gerrard et al., 2004).

Co-incubation of the cells with GCS inhibitors OGB-1 and OGB-2 did not reduce functional P-gp activity in these freshly isolated cells, in contrast to results observed in the P-gp over-expressing cell line VLB and normal MNC. OGB-2 is a more potent GCS inhibitor and this is reflected in a lower IC_{50} concentration and the degree of reduction of P-gp functional activity (Mellor et al., 2002). To compensate for the relative inefficiency of OGB-1, experimentally it was used at a much higher concentration than OGB-2, but still keeping within a clinically achievable and relevant range (van Giersbergen and Dingemanse, 2007). In spite of the lack of P-gp inhibition, pre incubation of freshly isolated CLL cells with OGB-1 or OGB-2 resulted in a reduced IC_{50} for cytotoxic drug CdA and cytostatic drugs chlorambucil and fludarabine in a proportion of the samples. Similar modulation of P-gp and sensitisation to daunorubicin with Z.3HCL was observed in AML cells (Gerrard et al., 2004).

Using the same OGB inhibitors, similar discordant findings were reported in cancer cell lines by Norris-Cervetto et al. (2004). They concluded that in the MCF7 modified cell line, inhibition of GCS using OGB inhibitors did not reverse MDR. It was suggested that the sensitisation achieved using other GCS inhibitors like PDMP and its analogues may be due to its non specificity to GCS (Bieberich et al., 1999; Liour and Yu, 2002). The possibility that OGB-1 and OGB-2 may act as substrates for P-gp was investigated by Norris-Cavetto et al. in 2006, and they concluded that both GCS inhibitors were not substrates for P-gp (Norris-Cervetto et al., 2006).

In conclusion, the GCS inhibitors OGB-1 and OGB-2 sensitise CLL cells to conventional cytotoxic and cytostatic drugs used in their treatment, but appear not to modulate P-gp functional activity in these cells.

Acknowledgements

We thank Dr Michael Burgess and Eli Lilly for providing Z.3HCL and funding this study. We also thank the Royal Free Hampstead NHS Trust Trustees for additional support towards this study.

References

Advani, R., Saba, H.I., Tallman, M.S., Rowe, J.M., Wiernik, P.H., Ramek, J., Dugan, K., Lum, B., Villena, J., Davis, E., Paietta, E., Litchman, M., Sikic, B.I., Greenberg, P.L., 1999. Treatment of refractory and relapsed acute myelogenous leukemia with combination chemotherapy plus the multidrug resistance modulator PSC 833 (Valspodar). Blood 93, 787–795.

Andersson, U., Butters, T.D., Dwek, R.A., Platt, F.M., 2000. N-butyldeoxygalactonojirimycin: a more selective inhibitor of glycosphingolipid biosynthesis than N-butyldeoxynojirimycin, in vitro and in vivo. Biochem. Pharmacol. 59, 821–829.

Beck, W.T., Cirtain, M.C., 1982. Continued expression of vinca alkaloid resistance by CCRF-CEM cells after treatment with tunicamycin or pronase. Cancer Res. 42, 184–189.

Bieberich, E., Freischutz, B., Suzuki, M., Yu, R.K., 1999. Differential effects of glycolipid biosynthesis inhibitors on ceramide-induced cell death in neuroblastoma cells. J. Neurochem. 72, 1040–1049.

Bohm, P., Kunz, W., Horny, H.P., Einsele, H., 2001. Adult Gaucher disease in association with primary malignant bone tumors. Cancer 91, 457–462.

Butters, T.D., Dwek, R.A., Platt, F.M., 2003. New therapeutics for the treatment of glycosphingolipid lysosomal storage diseases. Adv. Exp. Med. Biol. 535, 219–226.

Consoli, U., Santonocito, A., Stagno, F., Fiumara, P., Privitera, A., Parisi, G., Giustolisi, G.M., Pavone, B., Palumbo, G.A., Di Raimondo, F., Milone, G., Guglielmo, P., Giustolisi, R., 2002. Multidrug resistance mechanisms in chronic lymphocytic leukaemia. Br. J. Haematol. 116, 774–780.

Cox, T., Lachmann, R., Hollak, C., Aerts, J., Van Weely, S., Hrebicek, M., Platt, F., Butters, T., Dwek, R., Moyses, C., Gow, I., Elstein, D., Zimran, A., 2000. Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. Lancet 355, 1481–1485.

Dantzig, A.H., Shepard, R.L., Cao, J., Law, K.L., Ehlhardt, W.J., Baughman, T.M., Bumol, T.F., Starling, J.J., 1996. Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res. 56, 4171–4179.

Elstein, D., Hollak, C., Aerts, J.M., Van Weely, S., Maas, M., Cox, T.M., Lachmann, R.H., Hrebicek, M., Platt, F.M., Butters, T.D., Dwek, R.A., Zimran, A., 2004. Sustained therapeutic effects of oral miglustat (Zavesca, N-butyldeoxynojirimycin, OGT 918) in type I Gaucher disease. J. Inherit. Metab. Dis. 27, 757–766.

Ganeshaguru, K., Wickremasinghe, R.G., Jones, D.T., Gordon, M., Hart, S.M., Virchis, A.E., Prentice, H.G., Hoffbrand, A.V., Man, A., Champain, K., Csermak, K., Mehta, A.B., 2002. Actions of the selective protein kinase C inhibitor PKC412 on B-chronic lymphocytic leukemia cells in vitro. Haematologica 87, 167–176.

Gerrard, G., Payne, E., Baker, R.J., Jones, D.T., Potter, M., Prentice, H.G., Ethell, M., McCullough, H., Burgess, M., Mehta, A.B., Ganeshaguru, K., 2004. Clinical effects and P-glycoprotein inhibition in patients with acute myeloid leukemia treated with zosuquidar trihydrochloride, daunorubicin and cytarabine. Haematologica 89, 782–790.

Gottesman, M.M., Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2, 48–58.

Gouaze-Andersson, V., Cabot, M.C., 2006. Glycosphingolipids and drug resistance. Biochim. Biophys. Acta 1758, 2096–2103.

Green, L.J., Marder, P., Slapak, C.A., 2001. Modulation by LY335979 of P-glycoprotein function in multidrug-resistant cell lines and human natural killer cells. Biochem. Pharmacol. 61, 1393–1399.

Hunault, M., Zhou, D., Delmer, A., Ramond, S., Viguie, F., Cadiou, M., Perrot, J.Y., Levy, V., Rio, B., Cymbalista, F., Zittoun, R., Marie, J.P., 1997. Multidrug resistance gene expression in acute myeloid leukemia: major prognosis significance for in vivo drug resistance to induction treatment. Ann. Hematol. 74, 65–71.

Itoh, M., Kitano, T., Watanabe, M., Kondo, T., Yabu, T., Taguchi, Y., Iwai, K., Tashima, M., Uchiyama, T., Okazaki, T., 2003. Possible role of ceramide as an indicator of chemoresistance: decrease of the ceramide content via activation of glucosylceramide synthase and sphingomyelin synthase in chemoresistant leukemia. Clin. Cancer Res. 9, 415–423.

Jamroziak, K., Smolewski, P., Cebula, B., Szmigielska-Kaplon, A., Darzynkiewicz, Z., Robak, T., 2004. Relation of P-glycoprotein expression with spontaneous in vitro apoptosis in B-cell chronic lymphocytic leukemia. Neoplasma 51, 181–187.

Juliano, R.L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim. Biophys. Acta 455, 152–162.

Karaszi, E., Jakab, K., Homolya, L., Szakacs, G., Hollo, Z., Telek, B., Kiss, A., Rejto, L., Nahajevszky, S., Sarkadi, B., Kappelmayer, J., 2001. Calcein assay for multidrug resistance reliably predicts therapy response and survival rate in acute myeloid leukaemia. Br. J. Haematol. 112, 308–314.

Kok, J.W., Veldman, R.J., Klappe, K., Koning, H., Filipeanu, C.M., Muller, M., 2000. Differential expression of sphingolipids in MRP1 overexpressing HT29 cells. Int. J. Cancer 87, 172–178.

Krishnan, B., Ganeshaguru, K., Baker, R., Richfield, L., Mehta, A.B., 2003. Multidrug resistance and myelomonocytic leukaemia in Gaucher's disease. Haematologica 88, ECR02.

Lavie, Y., Cao, H., Bursten, S.L., Giuliano, A.E., Cabot, M.C., 1996. Accumulation of glucosylceramides in multidrug-resistant cancer cells. J. Biol. Chem. 271, 19530–19536.

Lavie, Y., Cao, H., Volner, A., Lucci, A., Han, T.Y., Geffen, V., Giuliano, A.E., Cabot, M.C., 1997. Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. J. Biol. Chem. 272, 1682–1687.

Leith, C.P., Kopecky, K.J., Chen, I.M., Eijdems, L., Slovak, M.L., McConnell, T.S., Head, D.R., Weick, J., Grever, M.R., Appelbaum, F.R., Willman, C.L., 1999. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood 94, 1086–1099.

Leonard, G.D., Fojo, T., Bates, S.E., 2003. The Role of ABC Transporters in Clinical Practice. Oncologist 8, 411–424.

Liour, S.S., Yu, R.K., 2002. Differential effects of three inhibitors of glycosphingolipid biosynthesis on neuronal differentiation of embryonal carcinoma stem cells. Neurochem. Res. 27, 1507–1512.

List, A.F., Kopecky, K.J., Willman, C.L., Head, D.R., Persons, D.L., Slovak, M.L., Dorr, R., Karanes, C., Hynes, H.E., Doroshow, J.H., Shurafa, M., Appelbaum, F.R., 2001. Benefit of cyclosporine modulation of drug resistance in patients with poor-risk acute myeloid leukemia: a Southwest Oncology Group study. Blood 98, 3212–3220.

Liu, Y.Y., Han, T.Y., Giuliano, A.E., Cabot, M.C., 1999. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. J. Biol. Chem. 274, 1140–1146.

Lucci, A., Cho, W.I., Han, T.Y., Giuliano, A.E., Morton, D.L., Cabot, M.C., 1998. Glucosylceramide: a marker for multiple-drug resistant cancers. Anticancer Res. 18, 475–480.

Matutes, E., Owusu-Ankomah, K., Morilla, R., Garcia, M.J., Houlihan, A., Que, T.H., Catovsky, D., 1994. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 8, 1640–1645.

Mellor, H.R., Nolan, J., Pickering, L., Wormald, M.R., Platt, F.M., Dwek, R.A., Fleet, G.W., Butters, T.D., 2002. Preparation, biochemical characterization and biological properties of radiolabelled N-alkylated deoxynojirimycins. Biochem. J. 366, 225–233.

Norris-Cervetto, E., Callaghan, R., Platt, F.M., Dwek, R.A., Butters, T.D., 2004. Inhibition of glucosylceramide synthase does not reverse drug resistance in cancer cells. J. Biol. Chem. 279, 40412–40418.

Norris-Cervetto, E., Butters, T.D., Martin, C., Modok, S., Dwek, R.A., Callaghan, R., 2006. Can P-glycoprotein influence the bioavailability of iminosugar-based glucosylceramide synthase inhibitors? Eur. J. Pharmacol. 530, 195–204.

Olshefski, R.S., Ladisch, S., 2001. Glucosylceramide synthase inhibition enhances vincristine-induced cytotoxicity. Int. J. Cancer 93, 131–138.
Oscier, D., Fegan, C., Hillmen, P., Illidge, T., Johnson, S., Maguire, P., Matutes, E., Milligan, D., 2004. Guidelines on the diagnosis and management of chronic lymphocytic leukaemia. Br. J. Haematol. 125, 294–317.
Platt, F.M., Neises, G.R., Dwek, R.A., Butters, T.D., 1994a. N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. J. Biol. Chem. 269, 8362–8365.
Platt, F.M., Neises, G.R., Karlsson, G.B., Dwek, R.A., Butters, T.D., 1994b. N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing. J. Biol. Chem. 269, 27108–27114.
Radin, N.S., 1999. Chemotherapy by slowing glucosphingolipid synthesis. Biochem. Pharmacol. 57, 589–595.
Tepper, A.D., Diks, S.H., van Blitterswijk, W.J., Borst, J., 2000. Glucosylceramide synthase does not attenuate the ceramide pool accumulating during apoptosis induced by CD95 or anti-cancer regimens. J. Biol. Chem. 275, 34810–34817.
Turzanski, J., Grundy, M., Shang, S., Russell, N., Pallis, M., 2005. P-glycoprotein is implicated in the inhibition of ceramide-induced apoptosis in TF-1 acute myeloid leukemia cells by modulation of the glucosylceramide synthase pathway. Exp. Hematol. 33, 62–72.
van Giersbergen, P.L.M., Dingemanse, J., 2007. Influence of food intake on the pharmacokinetics of miglustat, an inhibitor of glucosylceramide synthase. J. Clin. Pharmacol. 47, 1277–1282.
van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P., van Meer, G., 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 87, 507–517.
Veldman, R.J., Mita, A., Cuvillier, O., Garcia, V., Klappe, K., Medin, J.A., Campbell, J.D., Carpentier, S., Kok, J.W., Levade, T., 2003. The absence of functional glucosylceramide synthase does not sensitize melanoma cells for anticancer drugs. FASEB J. 17, 1144–1146.
Wagner, S.D., Cwynarski, K., 2004. Chronic lymphocytic leukaemia: new biological markers for assessing prognosis. Hematol. J. 5, 197–201.
Wishart, G.C., Bissett, D., Paul, J., Jodrell, D., Harnett, A., Habeshaw, T., Kerr, D.J., Macham, M.A., Soukop, M., Leonard, R.C., 1994. Quinidine as a resistance modulator of epirubicin in advanced breast cancer: mature results of a placebo-controlled randomized trial. J. Clin. Oncol. 12, 1771–1777.
Yusa, K., Tsuruo, T., 1989. Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. Cancer Res. 49, 5002–5006.