Clinical effects and P-glycoprotein inhibition in patients with acute myeloid leukemia treated with zosuquidar trihydrochloride, daunorubicin and cytarabine

[haematologica]
2004;89:782-790

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A B S T R A C T

Background and Objectives. P-glycoprotein (P-gp) is a major cause of multidrug resistance (MDR) in acute myelogenous leukemia (AML) and is thought to contribute to the failure of chemotherapy. Zosuquidar trihydochloride (Z.3HCL) is a potent and selective inhibitor of P-gp which rapidly and effectively inhibits drug efflux.

Design and Methods. The aim of this study was to evaluate the clinical effects of Z.3HCL and determine its influence on P-gp activity. Sixteen AML patients were entered into a phase 1 dose ranging clinical trial of Z.3HCL, co-administered intravenously with daunorubicin and cytosine arabinoside (ARA-C). Clinical outcomes, toxicity abd adverse events were assessed. P-gp function was analyzed by flow cytometry. *In vitro* cytotoxicity was studied using the MTT assay.

Results. Eleven patients achieved a complete remission and one a partial remission with a median survival of 559 (range 38-906) days. Non-hematologic grade 3 and 4 toxicities were seen in 4 patients. Z.3HCL infusion was associated with rapid inhibition of Rh123 efflux in $CD56^+$ cells in 16/16 patients and in $CD33^+$ cells from 6/10 patients. The median inhibition was 95% for $CD56^+$ cells and 85.25% for $CD33^+$ cells was significantly elevated in 6/16 patients. The median IC_{50}, using a MTT assay for daunorubicin, decreased significantly between Z.3HCL modulated and unmodulated cells (n=11,153 and 247 ng/mL respectively, p=0.01).

Interpretation and Conclusions. The modulator Z.3HCL is a specific inhibitor of P-gp efflux and can be given safely to patients with AML in combination with induction doses of conventional cytotoxic drugs.

Key words: zosuquidar trihydochloride, MDR assay, AML, P-gp.

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P-glycoprotein (P-gp) is a member of the ATP binding cassette (ABC) transporter superfamily, encoded by the *MDR1* (*ABCB1*) gene on the long arm of chromosome 7 (7q21.1).[1,2] It is thought to act as a flippase,[3] an ATP-dependant effector mechanism, intercepting and removing target compounds from the lipid bilayer, thus preventing intracellular (or intracompartmental) concentration of administered cytotoxic drugs. P-gp has a broad range of structurally diverse substrates which gives rise to the phenomenon of multidrug resistance (MDR), whereby a resistance mechanism engendered by a single cytotoxic entity is effective against a spectrum of structurally and functionally unrelated compounds.[4,5] P-gp is expressed on several human tissue types associated with secretory or barrier functions. P-gp can be upregulated in neoplastic cells in response to chemotherapy and this has been recognized as an obstacle to successful treatment.[6]

About one third of *de novo* cases of AML intrinsically overexpress P-gp at presentation and this frequency increases at relapse, in secondary AML, in the elderly and among patients with unfavorable cytogenetic patterns.[7] Overexpression of P-gp has been associated with reduction in the accumulation of cytotoxic agents and consequent ineffective killing of blast cells.[8,9]

P-gp can be modulated by a range of compounds that either block the protein's function by binding to it, such as vanadate,[10] or compete by acting as a high avidity substrate, such as verapamil (VPM).[11] Numerous *in vitro* studies have investigated the ability of these substances to modulate MDR, mostly using cell lines and fluorescent dye efflux flow cytometry.[12] Some of

Table 1. Demographics of the patients and Z.3HCL dosing schedules.

	Z.3HCL dose	*Age (Years)*	*Sex*	*Diagnosis*	*Cytogenetics*
1	200 mg/m² in 6h	44	F	*de novo* M5a	Complex
2	200 mg/m² in 6h	30	M	*de novo* M5	Normal
3	200 mg/m² in 6h	36	M	*de novo* M4	Trisomy 22 inv(16)
4	200 mg/m² in 6h	54	M	relapsed M4	Trisomy 21
5	300 mg/m² in 6h	53	M	refractory M1	t(14;21)
6	300 mg/m² in 6h	36	F	*de novo* M2	Trisomy 8
7	300 mg/m² in 6h	24	M	*de novo* M6	Normal
8	400 mg in 3h	54	M	relapsed M2	t(6;9)
9	400 mg in 3h	56	M	*de novo* M1	Normal
10	400 mg in 3h	47	M	*de novo* M5a	Normal
11	400 mg in 3h	63	M	relapsed M4	Normal
12	400 mg in 3h	25	M	relapsed M1	Trisomy 4
13	400 mg in 3h	69	M	*de novo* M1	Failed
14	400 mg in 3h	68	M	*de novo* M1	Normal
15	400 mg in 3h	52	M	*de novo* M2	Monosomy 7
16	400 mg in 3h	70	F	*de novo* M2	Trisomy 11 (X)

these compounds, such as cyclosporin A and its non-immunosuppressive analog, PSC833, have been evaluated in clinical trials[13,14] and are reported to induce undesirable pharmacokinetic interactions at concentrations necessary for P-gp modulation.[15,16]

Zosuquidar trihydochloride (Z.3HCL) is a potent and selective third generation inhibitor of P-gp.[17] It is a difluorocyclopropyldibenzosuberane which binds allosterically to P-gp and inactivates its efflux function (concentration of half-maximum inhibition, Ki= 59nM).[17,18] It is not a P-gp substrate and therefore retains its activity for a long period after its infusion has been completed. The exact inhibitory mode of Z.3HCL is still unclear, but it is known that it does not have a significant effect on other multidrug transporters such as MRP1 (multidrug resistance protein, ABCC1) or BCRP (breast cancer resistance protein, ABCG2).[19,20] This specificity means that Z.3HCL, unlike some previous modulators, does not have appreciable pharmacokinetic interactions with co-administered drugs, thus enabling unaltered dosing regimens.[21] Of course, specificity for P-gp is a limitation in situations in which other ABC transporters are overexpressed and this must be balanced against increased drug-drug interactions resulting from a lack of specificity. Daunorubicin and cytosine arabinoside (ARA-C) are antineoplastic drugs commonly used in the treatment of AML. Daunorubicin inhibits the synthesis of nucleic acids and has antimitotic and cytotoxic effects. It is an avid substrate for P-gp efflux.[9] ARA-C is an antimetabolite that kills cells during the S-phase of the cell cycle by inhibiting DNA polymerase. It has not been demonstrated to be a P-gp substrate.[22]

The primary objective of this trial was to determine doses of Z.3HCL that can be administered safely, with acceptable toxicity, as short intravenous infusions in combination with daunorubicin and ARA-C to patients with AML and myelodysplastic syndrome (MDS). The secondary objectives of the trial were to determine the pharmacokinetics of Z.3HCL (and its metabolites) with daunorubicin and daunorubicinol and to determine the relationship between plasma levels of Z.3HCL and P-gp inhibition *ex vivo*. This report presents the results of the primary objective and the relationship between plasma levels of Z.3HCL and P-gp inhibition *ex vivo* and *in vitro*.

Design and Methods

Patients' eligibility and characteristics

Adult patients (aged 18-80 years) with a confirmed diagnosis of *de novo* or relapsed AML or MDS were eligible for inclusion in a phase 1 dose ranging clinical trial of Z.3HCL. All patients had an Eastern Co-operative Oncology Group (ECOG) performance status of 2 or less. There was no restriction on sex or FAB type, with the exception of AML FAB type M3 (acute promyelocytic leukemia) patients, who were excluded from the trial because of associated coagulation complications and the preferred use of all-trans retinoic acid therapy.[23] Sixteen AML patients (FAB type[24] M1-5, M2-4, M4-3, M5-3 and M6-1) were entered; 13 males and 3 females with a median age of 52.5 years (range: 24 - 70 years). Six patients had normal cytogenetics, 9 had cytogenetic aberrations including 2 with an unfavorable pattern; cytogenetic analysis was unsuccessful in the remaining patient. The median white blood cell (WBC) count at the start of treatment was 3.4×10^9/L (range $0.9\text{-}45.4\times10^9$/L). The patients' demographics and inclusion and exclusion criteria are shown in

Table 2. Inclusion and exclusion criteria.

Inclusion criteria	*Exclusion criteria*
Morphologic diagnosis of AML or MDS	Acute promyelocytic leukemia
Prior chemotherapy completed >4 weeks previously	No chemotherapy in the last 4 weeks No previous study involving modulation of P-gp
Performance status 0-2[1]	Previous cytarabine-related neurotoxicity
Adequate organ function[2]	Unstable angina, uncontrolled atrial fibrillation or arrhythmias
Age 18-80 years	Serious concomitant disease
Negative pregnancy test and/or adequate contraception where appropriate	Pregnancy or breast-feeding

[1]Eastern Co-operative Oncology Group (ECOG); [2]Hepatic-bilirubin <1.5 times upper limit of normal (ULN) aspartate transaminase <2.5 times ULN. Renal-serum creatinine <1.5mg/L, or greater than calculated creatinine clearance >40 mL/min (calculated using Cockcroft and Gault formulae). Cardiac -2D echocardiogram with an ejection fraction of >45% .

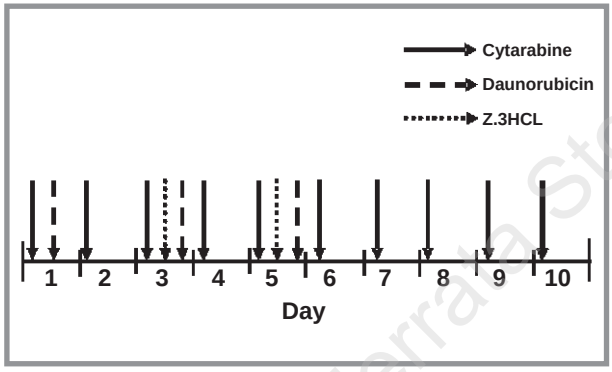

Figure 1. Chemotherapy dosing schedule. The schedule for ARA-C (200 mg/m²/d), daunorubicin (50 mg/m²/d) and Z.3HCL (as under study design and Table 1), all given by intravenous infusion.

Tables 1 and 2. The study was approved by the Royal Free Hospital Ethics Committee and all patients entered gave written informed consent to their participation.

Study design

ARA-C was infused via a central line at 200 mg/m² day for 7-10 days. Daunorubicin was infused at 50 mg/m² day by slow intravenous push on days 1, 3 and 5. Z.3HCL was given on days 3 and 5 prior to the daunorubicin, as either a 3 or 6h intravenous infusion to allow assessment of the effect of Z.3HCL on daunorubicin pharmacokinetics (Figure 1). The patients were divided into 3 dosing groups for Z.3HCL: 4 patients received 200 mg/m² in a 6h infusion, 3 received 300 mg/m² in 6 h, and 9 were given a fixed dose of 400 mg over 3 h (Table 1). The design is schematically represented in Figure 1. The fixed dose schedule was based on initial pharmacokinetic data derived from a phase I study in solid tumors.[25]

All patients entered into the study had adequate organ function (Table 2). Patients were monitored for response and adverse events continuously during their in-patient stay for 27 to 74 days and thereafter once or twice weekly depending on clinical status. Bone marrow aspirates were performed at 3-4 weeks when evidence of engraftment was obtained. Complete remission (CR, CR1, CR2) during follow-up was defined as a bone marrow aspirate with less than 5% blasts with subsequent normalization of blood counts prior to the next course of treatment. Partial remission was defined as the presence of bone marrow regenerating normal hematopoietic cells and containing 5-20% leukemic blasts cells. Toxicity and adverse events were assessed daily using the NIH-NCI Common Toxicity Criteria.[26] Follow-up was independent of the sponsor and the protocol. Pharmacokinetic analyses were performed on blood samples taken before and at various time intervals following the administration of Z.3HCL; the data have been presented and discussed elsewhere by Callies *et al.*[27]

Flow cytometric detection of P-gp function

Peripheral blood samples were taken from the patients using Becton Dickinson CPT tubes. These were taken, depending upon the Z.3HCL dosing group, at either 0, 1, 6, 8, 24 or 0, 0.5, 3, 6, 12 and 24 h and processed in batches of two. The CPT tubes were centrifuged at 1,500g (2,900 RPM) at ambient temperature for 20 minutes to separate the mononuclear white cell component through a density gradient gel. After three washes with HBSS, the cells were aliquoted into 2 sets of 3 tubes. One set of tubes was spiked with 100nM Z.3HCL, which would ensure maximal *in vitro* P-gp inhibition. The other set received RPMI to establish a control sample. The cells were then incubated with 50 ng/mL R123 (Molecular Probes, R-032) for 90min at 37°C, washed with ice-cold wash buffer (PBS + 0.1% BSA) and incubated for 15min at 37°C with anti-CD56 Cy-chrome antibody (Pharmingen, 31668X). After red cell lysis, (1 mL ddH_2O for 30 seconds followed by 1 mL ×2 PBS), fluorescence was read on a Becton Dickinson FACScan using CellQuest software, and the data analyzed and compensated with Verity Winlist 4.0 software. R123 is a P-gp substrate and the relative brightness of a cell read in a flow cytometer is a direct indication of the cell's accumulation function. Anti-CD33 labeling was used in parallel to anti-CD56 in the R123 assay for direct gating of blast cells when sufficient material was available.[28-30]

P-gp expression, calcein and drug sensitivity assays

Peripheral blood was collected in preservative free heparin. Mononuclear cells were separated by density gradient centrifugation on Lymphoprep (Nygaard, Norway). P-gp expression was studied on a FACScan (Becton Dickinson) using 500 ng/mL of the monoclonal antibody MRK16 (TCS Biologicals, UK). The blast cells were selected using forward scatter and side scatter gating. A discrete, monophasic fluorescent profile was obtained, indicating the selection of a homogeneous blast cell population. 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.[8,31] A MCF ratio of >1.1 was considered to indicate P-gp positivity. This value was derived from the mean of MRK16 ratios + 1SD from the multidrug activity-factor negative group of 53 AML samples studied as part of our on-going investigations (*data not shown*).

A functional P-gp accumulation assay using 50nM of the fluorescent dye calcein-AM with either Z.3HCL or 10 μM verapamil was used to assess *in vitro* P-gp activity. Once inside cells, calcein-AM is cleaved by cellular esterases and the calcein derivate is fluorescent. P-gp actively effluxes calcein-AM but not the derivative calcein.[32] The calcein-AM accumulation assay has been widely used to detect P-gp functional activity in AML cells.[33,34] The MCF was used to generate a multidrug activity factor (MAF), a ratiometric figure denoting the ability of each modulator to correct calcein efflux.

In vitro cytotoxicity was studied using the MTT assay at six concentrations of the cytotoxic drugs (ARA-C, daunorubicin, mitoxantrone, all from Aldrich-Sigma, UK). The cytotoxic drugs were studied either alone or in combination with 100 nM of Z.3HCL, a concentration that is not cytotoxic. Results are expressed as the concentration of drug that decreased cell viability by 50% in the MTT assay compared with the cell viability of controls incubated in the absence of drug (IC_{50}, calculated by Prism software).[35]

Statistical methods

Basic statistical inferences were performed using Microsoft Excel for Windows, which was also used to create the tables and Figure 1. Graphpad Prism 3.02 software for Windows was used to compute Student's T-tests and Wilcoxon non-parametric tests for the MTT assays, construct linear regression and correlations for the P-gp and calcein-AM data and to create all the graphs shown. Verity Winlist 4.0 software for Windows was used for the analysis and off-line compensation of the R123 FACS data. CELLQuest software for Macintosh OS9, by Becton-Dickenson, was used to acquire all FACS data.

Results

Clinical responses and toxicity

Sixteen patients were entered into this trial; 11 had *de novo* AML, 4 had relapsed AML and one had refractory AML. None of the patients had previously received any drug resistance-modifying treatment. Eleven patients achieved complete remission according to the criteria (defined in the Design and Methods section) including one patient whose bone marrow was regenerating haematopoietic cells and contained less than 5% blasts but who died of a probable pulmonary embolus prior to normalization of blood counts (ANC 1.7×10^9/L and platelets 31×10^9/L). One patient achieved a partial remission. The median survival for the 12 patients is 559 (range 38-906) days. Five of the 11 patients died later, 3 of progressive disease after a relapse and 2 of infection after a mini bone marrow transplant. All 6 patients still alive with a median survival of 722 (range 510-906) days in CR had *de novo* AML. There were 3 deaths prior to achieving CR/PR, 2 related to AML (both patients had poor risk cytogenetics) and one, as described above, of a probable pulmonary embolus. The median time to neutrophil recovery (>0.5×10^9/L) was 21 days and that to platelet recovery (>50×10^9/L) was 23 days.

Z.3HCL, in combination with daunorubicin and ARA-C, was well tolerated. Toxicities and adverse events were graded by NIH-NCI Common Toxicity Criteria and are shown in Table 3. Four patients experienced grade 3 and grade 4 toxicities; there were no on study deaths. Adverse events not attributed to Z.3HCL were mostly those predictable following chemotherapy. All patients experienced neutropenic fever, 7 had nausea and vomiting, 4 mucositis, 5 diarrhea and 6 abdominal pain. Palmar rash was observed in 6 patients (2 had both palmar and plantar rash) and 2 had plantar pain. One additional patient had palmar pain in the absence of any rash. Three other patients had a macular body rash, one had anorexia and one had peripheral edema. Significant laboratory abnormalities (hyperbilirubinemia) occurred in 2 patients. These abnormalities resolved spontaneously in both patients although in one case was reported as a severe adverse event. This patient also had renal failure as another severe adverse event; however, the patients' baseline renal and liver functions had been abnormal.

The main toxicities of note seen with the combination therapy with Z.3HCL were neurological. Two patients experienced grade 3 hallucinations or confusion during the 24h following the Z.3HCL infusion. These patients received only the first dose of Z.3HCL. The first (patient #8) was a patient with relapsed AML who developed vivid hallucinations, bad dreams and fatigue: he had never experienced similar symptoms

during his previous 4 courses of chemotherapy and anti-emetic therapy. The second (patient #14) described mildly distressing visual hallucinations. This patient had fever at the time of confusion which probably exacerbated the adverse event. Symptoms resolved completely after 24h in both patients.

In general, the incidence of palmar/plantar syndrome was high and it is possible that the Z.3HCL may have exacerbated this reaction. The frequency of abdominal pain was also high but this could be the result of enhanced monitoring in the trial setting. However, it was considered that the inclusion of Z.3HCL did not significantly increase the toxicity of the induction regime with all individuals having average times for both engraftment and inpatient stay.

Ex vivo effects on P-gp inhibition and expression

The zosuquidar plasma concentration-time curves for the 3 cohorts are shown in Figure 2. A direct concentration-effect relationship between Z.3HCL concentration and inhibition of R123 efflux in $CD56^+$ lymphocytes has been reported previously.[27] All patients achieved plasma concentrations in excess of the IC_{90} value, according to this relationship.

In the R123 assay P-gp activity was completely inhibited in the $CD56^+$ cells in all 16 patients, with a median percentage inhibition of 98% (range 73-111%) 0.5-1h after starting the Z.3HCL infusion. A similar inhibition of P-gp activity was also demonstrated at 2-3h (median 90.5%, range 63-106%) (Figure 3A), then the inhibiton decreased, as demonstrated by an increase in R123 MCF ratio after 8h. Similarly, the median percentage inhibition was 82.25% (range 64-105.5%) in $CD33^+$ cells at 0.5-1hr in 6 out of 10 patients in whom Z.3HCL had been demonstrated to induce P-gp efflux modulation. The modulation by

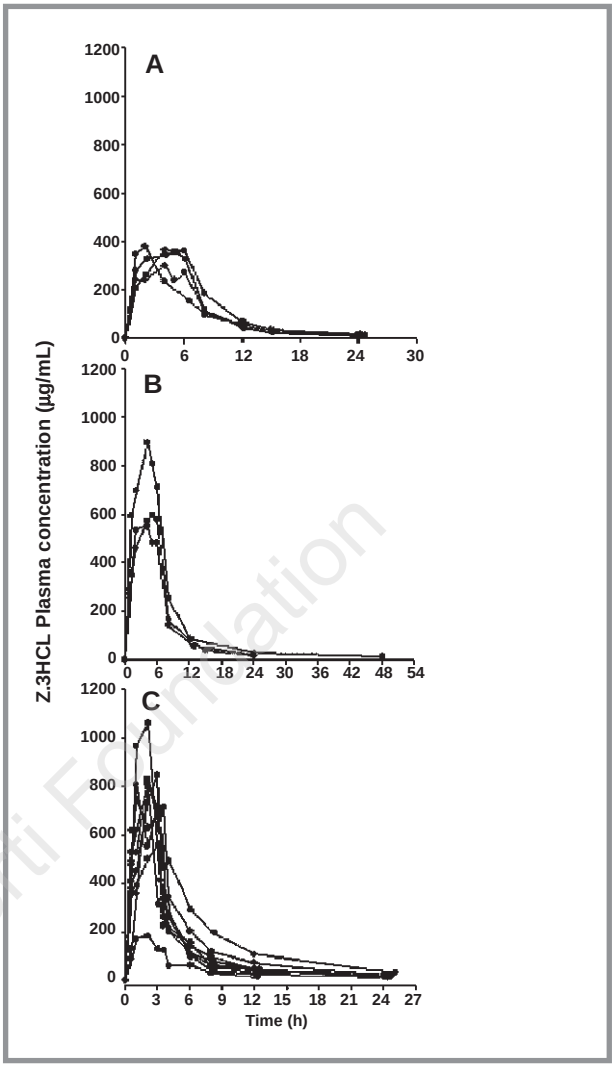

Figure 2. Z.3HCL plasma concentration-time curves. (A) The plasma concentration-time curves for Z.3HCL in the patients given the 3 different dose regimens. (B) 200 mg/m² in 6h. (C) 300 mg/m² in 6h and C. 400 mg/m² in 3h.

Table 3. Adverse events apparently associated with Z.3HCL.

	Patient 4 *200 mg/m² in 6h*	*Patient 6* *300 mg/m² in 6h*	*Patient 7* *300 mg/m² in 6h*	*Patient 8* *400 mg in 3 h*	*Patient 9* *400 mg in 3 h*	*Patient 11* *400 mg in 3 h*	*Patient 14* *400 mg in 3 h*
Hallucinations				Grade 3			Grade 3
Syncope	Grade 3			Grade 3			
Confusion/ vagueness		Grade 3					Grade 3
Short term memory loss			Grade 2				
Increased anxiety				Grade 3			
Hand tremor			Grade 1			Grade 1	
Abnormal dreams				Grade 3			
Lightheaded/ dizziness			Grade 2		Grade 2		
Vomiting				Grade 3			
Fatigue			Grade 2	Grade 3			

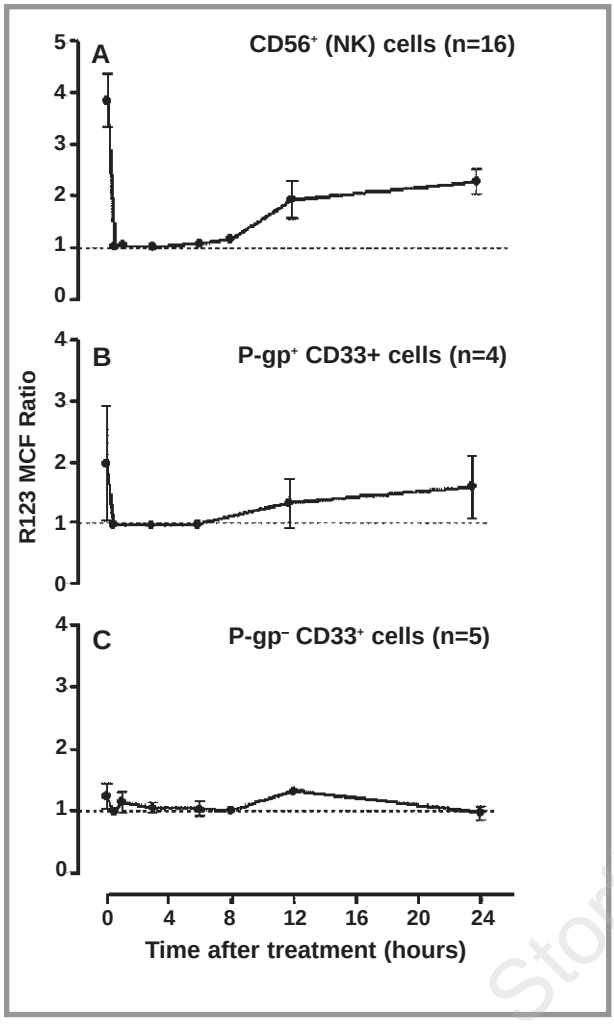

Figure 3. Mean P-gp inhibition Flow cytometric analysis of R123 efflux and its reversal by Z.3HCL measured *ex vivo* in patients' cells treated with Z.3HCL. Data expressed as efflux ratio over 24h. (A) CD56⁺ cells n=16, (B) CD33⁺ P-gp⁺ cells, n=4; and (C) CD33⁺ P-gp⁻ cells, n=5.

Z.3HCL in P-gp⁺ and P-gp⁻ cells is shown in Figure 3B and C, respectively. In the P-gp⁺ cells, there was a decrease in median modulation, similar to that seen in CD56⁺ lymphocytes, after 8h (Figure 3B). Blast cells from 6 of the 16 patients were P-gp⁺ according the MRK16 monoclonal antibody assay (MCF ratio >1.1, Table 4).

Calcein-AM accumulation assays

Blast cells isolated from 9/15 patients showed functional P-gp activity in that calcein-AM efflux was corrected (MAF≥10) by Z.3HCL with a median MAF of 13.4. Similarly, verapamil corrected calcein-AM efflux in 11/15 patients with a mean MAF of 18.7. Seven patients exhibited an efflux correction by both compounds (Table 4). The median calcein-AM efflux correction was greater in those cells that overexpressed P-gp (median MAF 37.9 for Z.3HCL and 35.4 for verapamil). A significant correlation was found between P-gp expression assessed by MRK16 and P-gp function assessed by calcein-AM efflux (Figure 4).

Table 4. Results of the *in vitro* studies.

Patient	*P-gp*	*Z.3HCL*	*Verapamil*	*Daunorubicin*	*Response*
1	–	+	+	–	CR1 - PD - Died
2	–	+	–	–	CR1
3	+	+	+	–	CR1
4	–	–	–	ND	PD - Died
5	–	ND	ND	ND	Fungal infection Died
6	–	–	–	ND	CR1
7	–	–	+	ND	CR1
8	–	–	+	–	CR2
9	+	+	+	+	CR1
10	–	+	–	–	CR1
11	+	+	+	–	PD - Died
12	–	+	+	+	CR1
13	–	–	+	–	PE - Died
14	+	+	+	+	CR1
15	+	+	+	ND	PD - Died
16	+	–	+	+	CR1
% Pos.	37.5 %	60.0 %	73.3 %	36.4 %	

P-gp expression (MRK16 assay); accumulation function (calcein-AM assay); and drug sensitivity (MTT assay). The corresponding initial response to treatment (CR: complete remission; PD: progressive disease; ND: not done) is also shown. PE: pulmonary embolism.

Drug sensitivity (MTT) assays

The median IC_{50} levels for daunorubicin differed significantly between cells unmodulated and modulated *in vitro* by Z.3HCL (n=11, median IC_{50}= 247 ng/mL control vs 153 ng/mL + Z.3HCL, p= 0.01) implying that P-gp-mediated efflux of daunorubicin can be reversed by Z.3HCL. Similarly, the median IC_{50} values for mitoxantrone were significantly lower in Z.3HCL-modulated cells (n=9, median IC_{50}=141 ng/mL control vs 57 ng/mL + Z.3HCL; p=0.04). There was no significant difference between the IC_{50} levels for ARA-C in cells that had or had not been treated with Z.3HCL (n=10, median IC_{50}=1571 ng/mL control vs 2313 ng/mL + Z.3HCL, p= 0.31) (Figure 5). Four out of eleven patients who showed sensitivity to daunorubicin achieved a CR (Table 4).

Discussion

In this phase 1 trial, patients with *de novo* and relapsed AML were treated with ARA-C, daunorubicin and escalating doses of the P-gp modulator, Z.3HCL. The results suggest that Z.3HCL is well tolerated in combination with these standard induction agents. The main toxicities and adverse events appear to be neurological. This is in keeping with the fact that P-gp is expressed in brain endothelial cells and may represent a direct effect of the drug, an effect of the con-

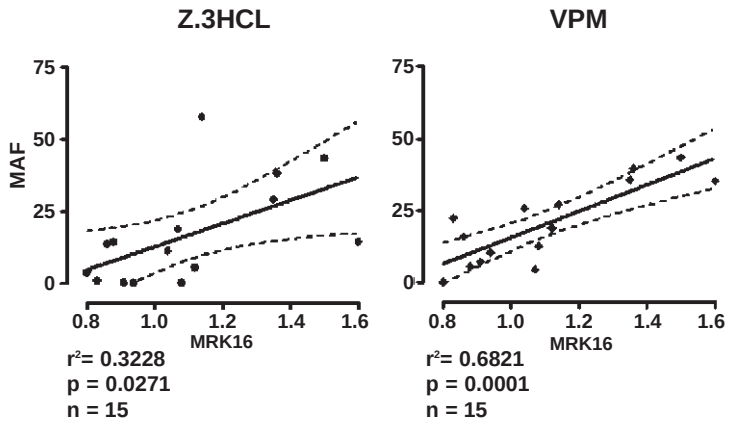

Figure 4. P-gp and calcein. The correlation between calcein accumulation by AML cells from 15 patients in the presence of either Z.3HCL or verapamil and the expression of P-gp. The broken line indicates 95% CI.

comitant cytotoxic drugs or an effect related to the inability to efflux naturally occurring toxins or metabolites. There is however no apparent correlation with plasma concentrations of Z.3HCL (Figure 2) or with the degree of P-gp inhibition as all patients achieved maximal inhibition in the R123 assay. Hence the possible mechanisms remain unknown. Previous studies of P-gp inhibitors such as verapamil, cyclosporin A and its analog PSC833 have been limited by direct toxicity or increased cytotoxic drug-related toxicities.[13-15] First generation P-gp inhibitors such as verapamil have low affinity for binding P-gp and consequently high doses are required to achieve complete P-gp inhibition thus leading to enhanced toxicity. In addition many of the P-gp inhibitors are themselves substrates for the pump and are therefore effluxed. This reduces the duration of the modulator's inhibition of pump activity once the drug's infusion has been completed. Our observation of a roughly half maximal inhibition of R123 efflux at 24h in $CD56^+$ cells from treated patients is in accordance with the direct nature of the pharmacokinetic/pharmacodynamic relationship.[25]

An additional adverse event that may occur with the *in vivo* use of P-gp modulators is enhanced toxicity of the co-administered cytotoxic agent. Although second generation P-gp inhibitors such as PSC833 and cyclosporin A have enhanced activity against P-gp, they also affect the pharmacokinetics of the cytotoxic agents with which they are administered.[12] Z.3HCL, on the other hand, was shown to have minimal effects on doxorubicin toxicity or pharmacokinetics in a phase I study in patients with advanced malignancies.[25]

Clinical studies examining the efficacy of P-gp inhibitors have been inconclusive. Although in principle, modulation of P-gp by various inhibitors should be beneficial, randomized phase III trials in AML do not demonstrate that this is unequivocally the case.[13-16] However, one study by the Southwest Oncology Group (SWOG) using cyclosporin A as the modulator, did support the concept that P-gp modulation in AML could improve survival.[36] The important difference between these modulation studies was that daunorubicin was given as a continuous intravenous infusion in the SWOG study, suggesting that the schedule of

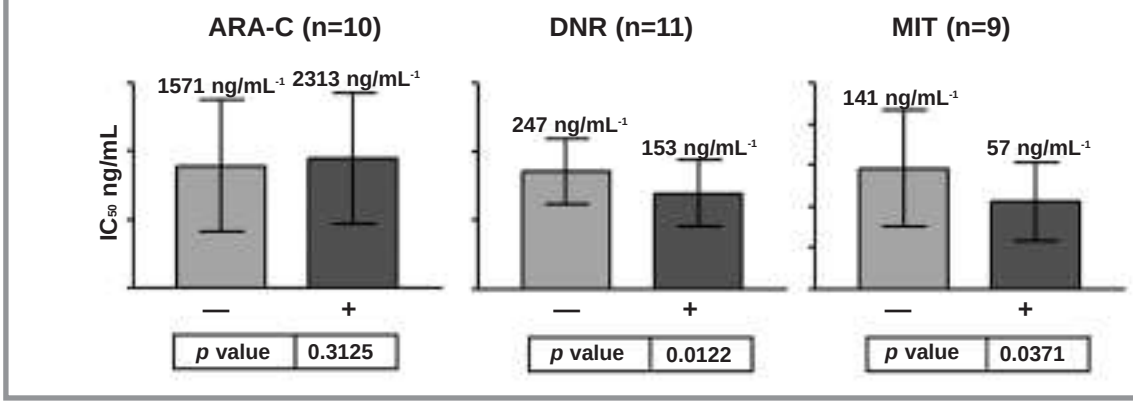

Figure 5. Median IC_{50}. The effect of Z.3HCL on IC_{50} levels of ARA-C (n=10), daunorubicin (n=11) and mitoxantrone (n=9) in AML cells treated *in vitro* over 72h with and without Z.3HCL using the MTT assay.

antineoplastic agent administration may be a determinant for successful modulation of P-gp in AML.

It has been suggested that although the bulk of AML cells do not overexpress P-gp, it is possible that P-gp$^+$ clonogenic AML blast cells may form a reservoir of resistant cells that contribute to treatment failure. It has been further suggested that this minor population of cells should be the target for P-gp modulation, as indeed was the case in this study which used infusions of Z.3HCL on the third and fifth days. This having been said, beneficial effects of modulation may not be reflected by CR rates but rather by disease-free survival or in the time to relapse.[37] However, studies by van der Pol *et al.*[38] show that emergence of minimal residual disease (MRD) in AML is not due to selection of pre-existing resistant subpopulations of cells.

The R123 accumulation data clearly show that Z.3HCL is a significant P-gp inhibitor in patients with AML. R123 efflux from CD56$^+$ cells was completely and rapidly inhibited in all of the patients studied, often by 0.5 hours after the start of infusion of the modulator. The loss of inhibitory effect observed at later time points may have been due to the method of processing the *ex vivo* samples. This may have lead to an underestimation of the *in vivo* potency of Z.3HCL. A similar trend was seen for the CD33$^+$ cells, confirming inhibition of P-gp in AML cells *in vivo*. However, CD33$^+$ cells tend to express significantly less P-gp than do CD56$^+$ cells and the inhibitory effect was, therefore, less pronounced. It is this inherently high P-gp expression and their relative abundance in peripheral blood that qualify CD56$^+$ cells as a convenient *ex vivo* surrogate marker for use in fluorescent dye accumulation assays measuring *in vivo* P-gp modulation.[29] Some AML blasts do express CD56, and since in most cases blasts cells express a much lower level of P-gp than do NK cells, this would have had the effect of reducing the sensitivity of the assay. On hindsight, inclusion of CD45 as a co-marker would have made the assay more robust. In a phase II trial with Z.3HCL, *ex vivo* studies on bone marrow myeloblasts also demonstrated successful modulation of P-gp efflux.[39]

The calcein-AM accumulation assay showed that Z.3HCL is as effective as verapamil at inhibiting functional P-gp efflux *in vitro*. Although more patients showed calcein-AM efflux correction with verapamil than with Z.3HCL, 5/6 P-gp$^+$ patients exhibited efflux correction with both modulators. The greater correction and better correlation shown by verapamil may be attributed to this modulator's ability to inhibit other drug transporters, including MRP1, while Z.3HCL specifically inhibits P-gp modulated efflux.[17] This lack of selectivity by verapamil can, however, result in deleterious changes in the pharmacokinetics of co-administered drugs, a problem not associated with Z.3HCL. The specificity of Z.3HCL for P-gp was further confirmed in a phase I trial in patients with advanced malignancies in whom clearance of the cytotoxic drug, doxorubicin, by other efflux pumps was not affected.[40,41] The clinical relevance of MRP1 expression as a significant resistance mechanism in AML is still being debated but its expression in this cohort of samples appears not to have contributed to the results (*data not shown*).

The combined drug IC_{50} levels show that co-incubating AML cells *in vitro* with Z.3HCL and cytotoxic drugs renders the AML cells from patients more sensitive to daunorubicin and mitoxantrone. Daunorubicin is a recognized substrate for P-gp and mitoxantrone can be effluxed by P-gp and the related half-transporter, BCRP.[20,40,41] Both drugs would, therefore, be removed from the cell by P-gp unlike AraC which is not a substrate; consequently the addition of Z.3HCL does not increase sensitivity to ARA-C. The reduction in the IC_{50} for daunorubicin confirms that Z.3HCL treatment in this cohort of patients would have inhibited the efflux of the infused daunorubicin from AML cells, thereby contributing to cell death and response to treatment.

Z.3HCL is a highly potent and specific inhibitor of P-gp efflux suitable for use as an adjuvant to chemotherapy. Its use may circumvent the development of multidrug resistance and overcome resistance in subpopulations of cells which are P-gp positive. It is significant that the dose of co-administered chemotherapy does not need to be altered. Z.3HCL is clinically well tolerated although there was a notable presence of neurological toxicities when it was given in combination with cytotoxic drugs. A randomized clinical trial is justified to assess the long-term safety and efficacy of Z.3HCL and to explore the concept of MDR modulation further.

GG, EP, RJB, DTJ, HM contributed to the collection/analysis of clinical/laboratory data and drafting the manuscript. MP, HGP, ME contributed to the analysis and interpretation of clinical data. KG, MB, ABM contributed to the conception, design, analysis, interpretation of data and critically reviewing the manuscript up to its final approval. The authors reported no potential conflicts of interest.

The clinical trial and GG were supported by Eli-Lilly

Manuscript received November 25, 2003. Accepted March 6, 2004.

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