Sequential Influences of Leukemia-Specific and Genetic Factors on P-Glycoprotein Expression in Blasts from 817 Patients Entered into the National Cancer Research Network Acute Myeloid Leukemia 14 and 15 Trials

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Abstract

Purpose: P-glycoprotein (Pgp) is a major prognostic factor for chemotherapy failure in acute myeloid leukemia (AML). This study compared the influence of genetic and leukemia-specific factors on Pgp.

Experimental Design: Eight hundred and seventeen samples were studied prospectively for Pgp protein expression and function and G1199A, G2677T, and C3435T polymorphisms in the encoding gene *ABCB1*.

Results: Age, low WBC count, high bcl-2, secondary AML and myelodysplastic syndrome, and adverse cytogenetics all correlated strongly with high Pgp (MRK16) protein expression. However, ABCB1 3435TT homozygosity was negatively correlated with Pgp. Pgp protein is only expressed in 41% of samples such that the negative effect of the polymorphism was not seen at baseline Pgp levels but was marked in the upper 41% of samples (MRK16 Δmean fluorescence intensity of 75th centile sample = 9 units for TT variant samples and 26 units for CC/CT; $P = 0.003$). However, no association was found between genetic factors and Pgp function using rhodamine 123 accumulation.

Conclusions: The genetic polymorphism 3435TT (which results in unstable mRNA) has a significant effect on Pgp expression, but this is only seen in ~40% of cases in which mRNA and protein are detectable. Moreover, leukemia-specific factors, such as low WBC count and poor risk cytogenetics, have a much greater effect than genetic polymorphisms on Pgp expression in AML blasts.

P-glycoprotein (Pgp) is a membrane transporter encoded by the multidrug resistance (*ABCB1*, *MDR1*) gene, which traps hydrophobic drugs in the plasma membrane of cells and effluxes them using an ATP-dependent process (1). Pgp has a broad range of substrates, including key chemotherapeutic drugs used in the treatment of acute myeloid leukemia (AML), notably anthracyclines and etoposide (2, 3). Pgp expression is detected in ~50% of AML blast samples (less in younger cohorts, more in older ones; ref. 2). All the major studies of adult AML concur that Pgp is a major predictor of chemoresistant disease (4).

Transcriptional regulation of Pgp expression is multifactorial, as the promoter has binding sites for a range of transcription factors (5). Polymorphisms in the *ABCB1* gene may also affect Pgp expression levels. There are at least 50 different *ABCB1* polymorphisms (6–9). The possibility that *ABCB1* polymorphisms can change the structure of Pgp and thus the specificity or efficiency of its pump function has been addressed in several studies. Notably, Kim et al. (7) noted low digoxin accumulation in cells with the T variant allele at position 2677. Woodahl et al. (10) found that the G1199A substitution increases accumulation of the substrate rhodamine 123 in epithelial cells. However, other studies found no evidence that these or other polymorphisms affected the efflux of a variety of substrates (11, 12). As the function of Pgp in excretory organs has a major influence on drug pharmacokinetics, there have also been several *in vivo* studies probing the role of polymorphisms. The 3435T variant has been linked to low Pgp expression and function in some reports, reviewed by Ambudkar et al. (9). Thus, the question of whether variant forms of Pgp induce changes in function is still questionable. The conflicting results in the literature may occur for several reasons (e.g., tissue-specific or disease-specific protein regulation,

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different effects on different substrates, small samples giving insufficient statistical power, or because artificial systems expressing particular individual polymorphisms do not recapitulate normal physiology in which some polymorphisms occur concurrently in linkage disequilibrium; ref. 7).

The working group on multidrug resistance in the United Kingdom National Cancer Research Network AML14 and AML15 trials is well placed to study the relationship between structure, function, and polymorphisms of Pgp in AML, as we have been measuring Pgp protein and function for 7 years in two centers and have results on 817 well-characterized presentation samples. This includes data on three single nucleotide polymorphisms: G2677T ($Ala^{893}Ser$), C3435T (synonymous), and G1199A ($Ser^{400}Asn$). G2677T and C3435T occur frequently and seem to be linked such that ~40% of cases in one AML study were heterozygous for both, 25% were wild-type for both, and 14% were variant for both (13). G1199A, which occurs in ~5.5% healthy Caucasians (14), was studied because it has been reported to decrease Pgp function (10) and has not yet been investigated in AML.

This study was designed to further our understanding of the factors that influence Pgp expression in AML blasts. We compare the effect of leukemia-specific factors (cytogenetics, WBC count, type of AML, and bcl-2 overexpression) and of genetic factors (*ABCB1* polymorphisms) and age on blast Pgp protein expression and function in a large cohort of the National Cancer Research Network trial AML patients.

Materials and Methods

Patient samples. Presentation bone marrow or peripheral blood samples from 817 patients entered into the United Kingdom AML14 (n = 323) and AML15 (n = 494) trials were taken into preservative-free heparin or into EDTA tubes following informed consent as approved by local and national ethics committees and sent to one of two laboratories in Nottingham and Cardiff over a 7-year period (1999-2006). AML14 was a trial for patients ages >60 years with AML or high-risk myelodysplastic syndrome (>10% blasts), which closed in 2006. AML15 is an ongoing trial predominantly for AML patients ages <60 years; however, fit patients ages ≥60 years can also be entered into AML15 and contributed 12% of the samples. Pgp was not measured in patients with M3 AML.

Because preliminary studies (data not shown) indicated that cells deteriorate after 48 h, only samples received within 48 h of being removed from the patient were analyzed. Mononuclear cells were isolated by standard density gradient techniques using Histopaque (Sigma-Aldrich) in the first step. After preliminary studies (data not shown) to check that fresh and frozen samples give similar results, some samples were analyzed fresh (particularly when hypocellular) and others were cryopreserved in liquid nitrogen and analyzed in batches. For functional assays, thawed samples were rested for 90 min before assay to allow time for recovery of function. Thawed and rested or fresh samples were then subjected to viability analysis using trypan blue exclusion. Only samples with >80% viable blasts were processed further. The study of Pgp protein and function was prospective such that these could be, and indeed frequently were, measured on samples with a low total mononuclear cell yield (as low as 3×10^6 total cells for a functional assay).

Flow cytometric methods for Pgp (phenotypic and functional) and bcl-2. Pgp protein and function were measured using flow cytometric methodology, described in detail elsewhere (15). Each assay involved labeling with the fluorescent probe or antibody of interest and with an antibody against CD45 to allow leukemic (CD45 low/side scatter low) cells to be gated (16, 17). The peridinin chlorophyll protein conjugate to CD45 was chosen to avoid spectral overlap with FITC labels. Briefly, Pgp substrate efflux modulation by PSC-833 was determined in an accumulation assay using rhodamine 123 (Sigma) based on the report by Broxterman et al. (18). For protein measurement, MRK16 anti-Pgp (Kamiya Biomedical) antibody (30 min, room temperature) was used followed by 20% normal rabbit serum to block (30 min, 4°C) and FITC-conjugated goat anti-mouse secondary antibody (30 min, 4°C; Dako). Bcl-2 was measured on 5×10^5 cells per tube, which had been fixed and permeabilized using the cell permeabilization kit from Harlan Seralab, followed by 5 μL directly FITC-conjugated anti-bcl-2 (15 min, room temperature; Dako). In 20 cases, samples that seemed viable by trypan blue exclusion at the start of assays showed a dying cell phenotype (low forward scatter, wide side scatter, and very low/negative CD45) on analysis. Results from these samples were excluded.

Obtaining reliable Pgp measurements in more than one reference laboratory. Protocols were based on methods that were found to be reproducible at a single laboratory during preliminary studies (19–21). Detailed standard operating procedures for the measurement of functional and phenotypic Pgp and potential pitfalls are described in detail elsewhere (15). In brief, both centers used the Calibrite bead system (Becton Dickinson) to set the voltages and compensation settings for the antibody assays and to monitor instrument performance at least weekly. Results were plotted over time to probe for abnormal trends. U937 (Pgp negative) and KG1a (Pgp positive) were used regularly as quality control. To adjust for potential differences between instruments, flow cytometric quantification was carried out using Dako fluorospheres to create standard curves and in-house software developed by Dr. T. Hoy was used to convert the channel numbers of the instrument to molecules of equivalent fluorochrome.

ABCB1 polymorphisms. DNA was extracted from cells using QIAamp blood DNA isolation kits (Qiagen) according to the manufacturers' protocol. cDNA was used in 23 cases where cells were not available for DNA extraction.

RFLP analysis was used for the genotyping of all three polymorphisms (G1199A, G2677T, and C3435T) as described previously (14). The frequency of the 2677A genotype in a healthy population is reported as only 1.9% (14): it was therefore impractical to study the effect of this polymorphism on Pgp expression in AML. Our assay did not distinguish between G and A genotypes such that results reported as G2677 are also expected to include a small number of A2677 genotype. Approximately 50 ng DNA was amplified using 2 units of AmpliTaq Gold (PE Applied Biosystems) in each PCR. Reactions were done on a PTC-100TM machine (MJ Research, Inc.) for 35 cycles using an annealing temperature of 57°C for 2677 and 3435 polymorphisms and 55°C for 1199 polymorphism. PCR products were assessed for quality and quantity and purified using QIAquick PCR Purification kit (Qiagen) before digestion.

The *ABCB1* gene is located on chromosome 7, which may be deleted in AML. The question of whether the RFLP results reflect genomic or leukemic DNA in these cases has been addressed by Illmer et al. (13) and by van den Heuvel-Eibrink et al. (22). Neither was able to show leukemia-derived hemizygosity, and the issue was not further addressed in our study. In preliminary analysis, exons 11, 21, and 26 were sequenced to establish positive controls, which were included in all batches of digests. Random cases totaling 5% of all those studied were sequenced during the course of the study to confirm RFLP results.

Real-time ABCB1 expression. AML blasts were enriched by CD2 depletion (using anti-CD2-coated Dynabeads) before RNA preparation using QIAamp RNA kits with DNase treatment according to the manufacturers' instructions (Qiagen). Up to 2 μg of RNA were used in a reverse transcription reaction with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers (Amersham Pharmacia). Quantitative PCR was done on an ABI Prism 7700 (PE Applied Biosystems) using Excite Real-time Mastermix with SYBR Green Detection (BioGene). Each reaction consisted of 1× Excite mastermix, SYBR green (1:60,000 final concentration), 40 nmol/L of

Table 1. Patient characteristics

	Median or number (%)
No. patients in study	817
Pgp protein expression (n = 651)	
Negative/low	381 (58)
High	270 (41)
Pgp function (n = 710)	
Negative	411 (58)
Low	165 (23)
High	134 (19)
Median age in years (n = 817)	58 (range, 8-87)
Median WBC $\times$ 10^9/L (n = 745)	13.5 (range, 0.5-311)
Type of AML (n = 802)	
De novo	672 (84)
Secondary	89 (11)
High-risk MDS	41 (5)
Cytogenetic-risk group (n = 635)	
Good risk*	73 (11)
Standard risk	461 (73)
Poor risk	101 (16)
G1199A (n = 614)	
GG	577 (94)
GA	35 (6)
AA	2 (0.5)
G2677T (n = 611)	
GG	203 (33)
GT	281 (46)
TT	128 (21)
C3435T (n = 617)	
CC	140 (23)
CT	285 (46)
TT	191 (31)
Median bcl-2 † (n = 528)	9.3 (range, 1.0-50.7)

Abbreviation: MDS, myelodysplastic syndrome.
*As acute promyelocytic leukemia patients were excluded, this group comprises t(8;21) and inv(16) patients only.
† Test/control antibody molecules of equivalent fluorochrome ratio.

both forward and reverse primers (ABCB1-qFOR, GCCACGTCAGCTCTGGATACAGAAAGTG; ABCB1-qREV, GTGCCATGCTCCTTGACTCTGCC), 1.6 μL DNA (or H_2O), and H_2O to a final volume of 20 μL. Thermal cycler conditions included incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Following the 40 cycles, the products were heated from 60°C to 95°C over 20 min to allow melting curve analysis to be done. This allowed the specificity of the products to be determined (single melting peak) and confirmed the absence of primer dimers.

To enable the levels of transcripts to be quantified, standard curves were generated using serial dilutions of KG1a cDNA. The housekeeping gene *β2-microglobulin* was used to standardize the samples (23) and the relative expression levels of *ABCB1* transcripts were therefore calculated as the ratio between the level of *ABCB1* and the level of *β2-microglobulin*. Negative controls (no template) were included in each experiment and all reactions were run in triplicate.

Statistics. Pgp expression and function in polymorphic groups were compared initially by χ^2 ANOVA. Significant two-way comparisons ($P < 0.05$) were further explored by Mann-Whitney tests. All P values are two sided. All analyses were done using Statistical Package for the Social Sciences software.

Cytogenetics. Stratification into favorable, intermediate, and adverse cytogenetics groups was made according to guidelines established in previous Medical Research Council studies (24). All cytogenetics reports were collected and reviewed by the Leukaemia Research Cytogenetics Group (25). As acute promyelocytic leukemia patients were excluded from the analysis, good risk was defined as the presence of t(8;21)(q22;q22) or inv(16)(p13q22).

Results

Obtaining reliable Pgp measurements in more than one reference laboratory. The literature suggests that obtaining intercenter agreement when measuring drug resistance variables has been problematic in the past and is more likely to succeed if the same assay protocols are used by participating laboratories and if both functional and phenotypic Pgp are measured (26, 27). Measures to optimize and confirm reproducibility in our study are described in Materials and Methods. Preliminary intercenter reproducibility studies of the first 60 samples from each center showed that the fluorescence distributions obtained for functional Pgp as well as for bcl-2 measurements showed good intercenter agreement, and the functional assay reproducibility data have been published (28). Pgp was recorded as functional when the PSC ratio was $\geq$1.7 based on a previous study (20). Pgp function was reported as high when it exceeded 3.4. The complete data distributions from the two centers were reanalyzed at the end of the current study and confirmed that the distributions of bcl-2 and functional Pgp were not significantly different between centers (data not shown). Regarding the MRK16 Pgp results, the two centers were able to distinguish the same cases as high or low in 13 of 15 (87%) cases but the results on a quantitative scale were insufficiently reproducible to be pooled. Therefore, the results were recorded as above or below the pilot study mean for each center individually before being combined. For this report, these values are referred to as "high" or "negative/low," but it should be borne in mind that there is no logical cutoff point between positive and negative Pgp protein expression in patient samples.

Incidence of phenotypic and functional Pgp and ABCB1 polymorphisms. Patient characteristics are summarized in Table 1. Six hundred and twenty patients had sufficient viable cells for comparison of functional and phenotypic Pgp. Of these, 30.0% were positive for both functional and phenotypic Pgp, 11.0% seemed to have phenotypic Pgp only, 9.3% had functional Pgp only, and 49.7% had negative/very low levels of both. The incidence of the G1199A, G2677T, and C3435T polymorphisms is also shown in Table 1. The three polymorphisms are all in Hardy-Weinberg equilibrium (data not shown).

Polymorphic haplotypes. The G2677T and C3435T polymorphisms have previously been shown to be linked (7, 13). The haplotype data from our study are shown in Table 2. It can be seen that the three most common haplotypes are 2677GG/3435CC, 2677GT/3435CT, and 2677TT/3435TT: these combinations comprise 73.6% of cases and therefore, similar to previous studies, strongly suggest linkage disequilibrium (7, 13).

Table 2. Haplotype analysis

G2677T	C3435T	No. patients (%)
GG	CC	123 (20.2)
GG	CT	65 (10.7)
GG	TT	14 (2.3)
GT	CC	13 (2.1)
GT	CT	208 (34.2)
GT	TT	58 (9.5)
TT	CC	1 (0.2)
TT	CT	9 (1.5)
TT	TT	117 (19.2)

Association of biological and genetic factors with phenotypic and functional Pgp. The factors that affect Pgp protein expression and function are shown in Table 3. It can be seen that both Pgp protein and function are strongly correlated with a high median age, low WBC, high bcl-2, secondary AML, and an adverse cytogenetic-risk group. In addition, the presence of the 3435TT variant polymorphism is associated with a lower Pgp protein compared with the CT and CC genotypes, although no correlation was seen between polymorphic groups and Pgp function. Given the striking association between Pgp overexpression and a relative low WBC count, the question arose of whether Pgp is directly associated with a low proportion of cycling cells and we present supporting data in Supplementary Fig. S1 to indicate that Pgp is indeed associated with a low proportion of cells observed to have >2n DNA on propidium iodide staining. Pgp has previously been associated with abnormal cytogenetics, particularly +8, -7/7q-, 5q-, and multiple abnormalities, compared with patients with good risk or normal cytogenetics (29–31): our data support these findings. The *ABCB1* gene coding for Pgp is on chromosome 7. A study using fluorescence *in situ* hybridization analysis of blasts with monosomy 7 confirmed that only one copy of the *ABCB1* gene is present in these cells (32). Despite this, patients with monosomy 7 have been reported to overexpress Pgp (31, 32). In keeping with these findings, high Pgp expression was found in 77% of monosomy 7 patients in our study.

In contrast with the report of Illmer et al. (13), we found no significant associations between adverse cytogenetics and polymorphisms, either individually or as common haplotypes (data not shown). We also examined the distribution of polymorphic variants with the main cytogenetic subgroups, inv(16), t(8;21), +8, -7, and/or complex karyotype, and found no atypical distributions (data not shown).

In addition, we studied whether any factors may predict Pgp protein and function in patients with intermediate-risk cytogenetics. Supplementary Table S1 shows that age, low WBC, and high bcl-2 all remain strongly correlated to the expression and function of Pgp in this subgroup. There were no significant correlations with polymorphisms in the intermediate-risk subgroup, although there was a trend toward variance for the C3435T polymorphism, with heterozygotes having higher Pgp expression than either of the homozygous groups ($P = 0.06$).

Genetic factors associated with Pgp expression and function when measured on a quantitative scale. Several previous studies in AML have shown that Pgp is expressed in 35% to 70% of adult cases (2). We reasoned that the effect of the 3435

Table 3. Factors associated with Pgp expression and function

Variable	Negative/low Pgp protein, number or median (%)	High Pgp protein, number or median (%)	*P*	Negative Pgp function, number or median (%)	Low Pgp function, number or median (%)	High Pgp function, number or median (%)	*P*
Median age (y)	56, *n* = 381	63, *n* = 270	<0.001	57, *n* = 411	58, *n* = 165	63, *n* = 134	0.001
Median WBC ($\times 10^9$/L)	23.9, *n* = 344	10.4, *n* = 254	<0.001	23.5, *n* = 369	9.9, *n* = 154	5.4, *n* = 123	<0.001
Type of AML							
De novo AML	338 (63)	200 (37)		364 (62)	126 (21)	97 (16)	<0.001
Secondary AML	27 (39)	43 (61)		27 (37)	22 (30)	24 (33)	
High-risk MDS	13 (34)	25 (66)	<0.001	17 (44)	12 (31)	10 (26)	
Cytogenetic-risk group							
Good risk	40 (63)	23 (36)		33 (51)	29 (45)	3 (5)	<0.001
Standard risk	235 (63)	139 (37)		259 (64)	76 (19)	69 (17)	
Poor risk	29 (36)	51 (64)	<0.001	29 (34)	26 (31)	30 (35)	
Main cytogenetic subgroups*							
inv(16)	22 (61)	14 (39)	Not determined	22 (63)	11 (31)	2 (6)	Not determined
t(8;21)	18 (67)	9 (33)		12 (40)	18 (60)	0 (0)	
Normal	176 (67)	88 (33)		193 (68)	54 (19)	37 (13)	
-5/5q-/-7/7q-	19 (35)	36 (65)		18 (31)	18 (31)	23 (39)	
-7	7 (23)	23 (77)		7 (21)	13 (39)	13 (39)	
+8	18 (39)	28 (61)		19 (37)	16 (31)	16 (31)	
complex	17 (34)	33 (66)		20 (37)	19 (35)	15 (28)	
C3435T							
CC	69 (63)	40 (37)	CC/CT vs TT	66 (57)	26 (22)	24 (21)	Not significant
CT	132 (57)	99 (43)	0.028	159 (65)	54 (22)	31 (13)	
TT	101 (70)	44 (30)		104 (65)	29 (18)	26 (16)	
G2677T							
GG	100 (64)	57 (36)	Not significant	99 (59)	39 (23)	31 (18)	Not significant
GT	136 (60)	92 (40)		155 (65)	56 (23)	29 (12)	
TT	62 (64)	35 (36)		72 (67)	14 (13)	21 (20)	
G1199A							
GG	284 (63)	168 (37)	Not significant	309 (63)	101 (21)	78 (16)	Not significant
GA	17 (57)	13 (43)		19 (68)	7 (25)	2 (7)	
AA	0	2 (100)		0	1 (50)	1 (50)	
Median bcl-2 †	8.5, *n* = 314	10.4, *n* = 208	<0.001	8.2, *n* = 304	9.7, *n* = 107	12, *n* = 98	<0.001

*These are not hierarchical groupings (i.e., some patients appear in more than one group).
†Test/control antibody molecules of equivalent fluorochrome ratio.

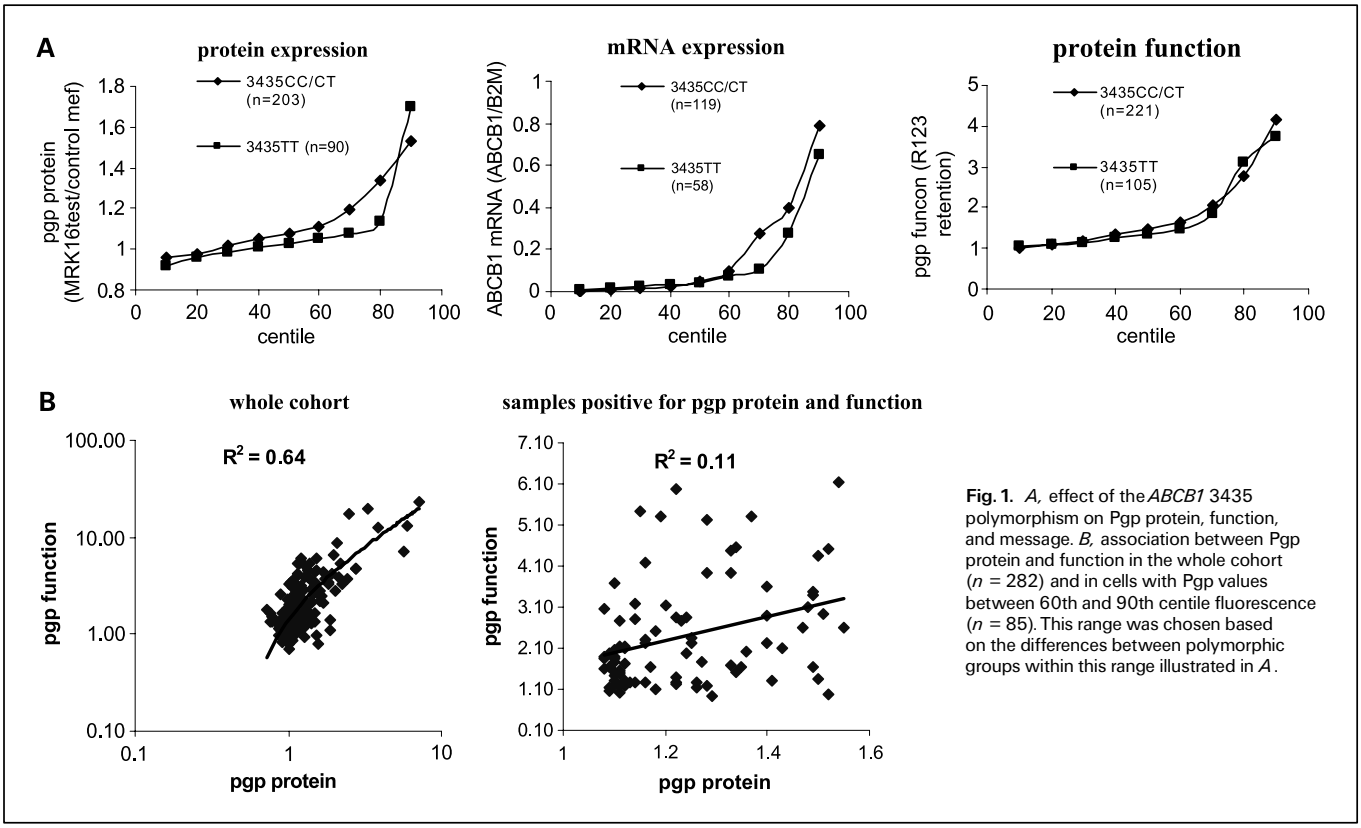

Fig. 1. *A,* effect of the *ABCB1* 3435 polymorphism on Pgp protein, function, and message. *B,* association between Pgp protein and function in the whole cohort (n = 282) and in cells with Pgp values between 60th and 90th centile fluorescence (n = 85). This range was chosen based on the differences between polymorphic groups within this range illustrated in *A*.

polymorphism on Pgp protein noted in Table 3 would be indeterminable in those cases where *ABCB1* gene transcription is not turned on but could be pronounced in cases where it is turned on. To obtain more sensitive information on the effects of *ABCB1* polymorphisms on Pgp expression and function, we needed to measure data obtained on a continuous scale. As discussed in the first part of the Results section, small differences in protein results obtained in the two centers meant that the results could be pooled when dichotomized but not when expressed on a continuous scale. Therefore, this part of the analysis was done using Pgp protein as a continuous variable in the cohort from the center that had the largest and most complete data set (294 samples had both protein and polymorphism data in this subgroup). We plotted Pgp centile values according to 3435 polymorphic group (Fig. 1A). The data showed that the effect of the 3435TT polymorphism was greatest at around the 75th centile. This implies that the effect of the polymorphisms is only seen in cases where one would expect high expression. Mann-Whitney tests (Table 4) confirmed significant differences between polymorphic groups when Pgp is measured on a quantitative scale. The data showed that, in addition to the 3435TT polymorphism, the 2677TT and the common variant haplotype had a significant effect on Pgp protein expression, with more marked differences at the 75th centile expression than at the median. To interpret Table 4, one should bear in mind that flow cytometric fluorescence values are reported relative to the isotype control; thus, if no Pgp is present, the value is 1. Therefore, the reported values at the 75th centile of 1.09 for the TT group, 1.24 for the CC group, and 1.27 for the CT group indicate relative fluorescence (Δmean fluorescence intensity) of 9, 24, and 27, units respectively.

These results led us to pose the question of whether the comparatively low protein expression seen in the 3435TT group was due to low mRNA expression. We therefore measured *ABCB1* mRNA by real-time PCR in the 177 patients from our cohort on whom cDNA was available. Preliminary analyses showed that Pgp surface protein, *ABCB1* mRNA expression, and Pgp function all strongly correlated, with P values of <0.001 (data not shown). When the medians and 75th centiles of the three 3435 genotypes were examined, the heterozygote genotype was associated with the highest median mRNA expression followed by the variant homozygote and then the wild-type homozygous genotype (Fig. 1A; Table 4). As in the case of protein, differences between polymorphic groups were more marked at 75th centile than at median expression, indicating that this pattern affects RNA as well as protein levels. However, at the mRNA level, the low expression in the 3435TT group was not statistically significant possibly due to a smaller number of samples in the analysis. The same pattern was observed for the 2677 polymorphism and for the 3435/2677 haplotype.

We then turned our attention to Pgp function. As shown in Fig. 1A, C3435TT and 2677TT variant homozygosity had no effect on the rhodamine efflux function of Pgp. At first, this finding seemed puzzling. However, the data illustrated in Fig. 1B may provide an explanation: a strong correlation between Pgp protein expression and Pgp function in the cohort as a whole (R^2 = 0.64) is predicated on the fact that most samples are either positive for both protein and function or negative for both protein and function. If, however, we ignore

Table 4. Effect of *ABCB1* polymorphisms on Pgp expression, Pgp function, and *ABCB1* mRNA

	Pgp expression (n = 294)		Pgp function (n = 327)		*ABCB1* mRNA (n = 177)	
	50th centile	75th centile	50th centile	75th centile	50th centile	75th centile
G2677T						
GG	1.07	1.23	1.47	2.65	0.036	0.311
GT	1.08	1.25	1.40	2.20	0.055	0.365
TT	1.04	1.13	1.36	2.23	0.048	0.169
	P = 0.017, GG+GT vs TT					
C3435T						
CC	1.07	1.24	1.52	2.59	0.027	0.269
CT	1.08	1.27	1.44	2.28	0.063	0.373
TT	1.03	1.09	1.34	2.10	0.043	0.159
	P = 0.003, CC+CT vs TT					
2677/3435						
All others vs	1.08	1.25	1.44	2.31	0.050	0.349
TT/TT	1.03	1.10	1.34	2.13	0.042	0.117
	P = 0.005, TT/TT vs others					

the negative data, and just examine those samples where differences in protein expression between polymorphic groups were marked (i.e., between the 60th and 90th centiles of Pgp expression), it can be seen clearly that the amount of Pgp protein is only a very weak predictor of the degree of rhodamine function (R^2 = 0.11). This therefore offers an explanation as to why the effect of the polymorphism is not seen at the functional level.

For further evidence of the extent to which Pgp expression in AML is regulated at a leukemia-specific or a genetic level, we measured Pgp protein on $CD56^+/CD45^{high}$ lymphocytes (natural killer cells) in patient samples and noted a correlation with natural killer cell Pgp only in the blast Pgp-positive cases (Supplementary Fig. S2; r = 0.58; P = 0.02); that is, these data reinforce the pattern that there are leukemia-independent influences on the level of Pgp expression in AML blasts only in those cases where leukemia-specific factors are allowing Pgp to be expressed.

G1199A polymorphism. For the G1199A polymorphism, 614 samples were studied to identify three genotypes. As a rare polymorphism, 93.8% of the studied population was wild-type (GG) at position 1199, whereas the heterozygote 1199GA accounted for 5.7% and only three patient samples were recognized as carriers of 1199AA genotype. The 1199A (variant) and wild-type genotypes of 2677 and 3435 were linked (P = 0.005 and 0.003, respectively). Because only three cases of 1199AA were found, Pgp expression and function analysis were compared in patients with wild-type (1199GG) at this position versus the patients with at least one copy of the A allele (1199A*).

The distribution for both groups for *ABCB1* transcript expression, Pgp expression, and Pgp was not significantly different when divided into 1199GG 1199A* according to Mann-Whitney test (this was irrespective of whether protein and function were measured on a continuous or on a dichotomized scale; data not shown).

Discussion

This study of factors influencing Pgp overexpression in AML blasts was designed to further our understanding about the level of Pgp regulation (i.e., leukemic or genetic) in AML. We have brought together leukemia-specific and genetic data and have shown the dominance of leukemia-specific factors (i.e., type of AML, WBC, and poor risk cytogenetics). These factors determine whether Pgp expression is turned on or off. The novel data from our polymorphism analysis, supported by the blast/natural killer comparison, indicate that only if Pgp is turned on do genetic factors secondarily affect expression levels.

This study emphasizes the importance of understanding the contribution of genetic factors to protein expression before examining downstream clinical variables: our results strongly suggest that polymorphisms could only be clinically significant in terms of blast behavior in patients who express Pgp. However, *ABCB1* polymorphisms in all patients could also affect the outcome of AML through their influence on nonblast tissues and pharmacokinetics. There have been two previous studies: one that found an association between *ABCB1* mRNA expression levels and the 2677 and 3435 polymorphisms in 136 patients (13) and the other finding no association between polymorphisms, protein, and function in 150 patients (33). Our data reveal that, even with the very large cohort we describe in Table 3, the relationship between Pgp expression and polymorphisms is weak (for 3435) or statistically nonsignificant (for 2677) when the whole cohort is analyzed. It is only in the upper 40% where Pgp is being turned on (Fig. 1), and particularly around the 75th percentile, that we see the effect of 3435 and 2677 polymorphisms on blast Pgp expression. We suggest that these novel findings are attributable to the design of our study: (*a*) large sample size and (*b*) the study was prospective and therefore variables were measured on fresh samples in hypocellular samples that often express high levels of Pgp—it is possible that these samples may be missed in retrospective studies.

There was a striking association in our study between a high WBC count and low Pgp expression and function. Bcl-2 is also associated with Pgp overexpression and could help account for the apoptosis-resistant phenotype we have previously noted in Pgp-positive AML blasts (34). The data in Supplementary Fig. S1 on 40 consecutive samples support our contention that the low WBC count associated with Pgp may be due to a low rate of cycling in Pgp-positive samples. Pgp is a well-described prognostic factor, but the point we wish to emphasize with

respect to the current data is that this may not simply be due to substrate drug efflux: cells that are cycling at a low rate are also comparatively resistant to antimetabolic non-Pgp substrate drugs, such as cytarabine (35). The strong correlations in the current study indicate that bcl-2 and a low rate of cycling may all contribute to multidrug resistance in Pgp-positive AML.

In common with several other studies in both normal and abnormal tissues (9), we have shown low Pgp expression in subjects with the homozygous 3435TT polymorphism. 3435T is a noncoding polymorphism, and until recently, it was generally thought that its predictive value might be due to its linkage disequilibrium with other factors. However, it has now been shown that the 3435 C to T substitution decreases mRNA stability (36). The reason why heterozygotes should have the highest mRNA and protein levels [also noted by Illmer et al. (13)] remains to be clarified: it is possible that the C allele may overcompensate for the low expression associated with the T allele. Another puzzle that remains to be resolved is the evidence that at the highest levels of Pgp protein expression (Fig. 1) the 3435TT variant curve crossed the CC/CT curve between the 80th and 90th centiles. As with the highest expression in heterozygotes referred to above, this could also suggest a compensatory mechanism. However, it could also be a statistical anomaly, given that the actual number of 3435TT variants above the 80th centile is only 18.

In quantitative analysis, the TT2677 polymorphism and the 2677TT/3435TT haplotype showed similar patterns to the TT3435 polymorphism, with greater differences between genotypes seen at the 75th centile than at the median. It is clear from Table 1 that there is a higher incidence of 3435TT than of 2677TT homozygotes, and because 92% of cases with 2677 variant homozygosity are also 3435TT variants (Table 2), it is unclear whether the 2677 polymorphism makes a unique contribution to Pgp expression levels. It is likely that the patterns of mRNA and protein expression noted for 2677 polymorphic variants in Table 4 are heavily influenced by linkage disequilibrium with the 3435 position: in the report by Wang et al. (36), there was no indication that 2677 variants were directly involved in mRNA stability. There has also been a report in which cDNA for variant *ABCB1* polymorphisms, both individually and as haplotypes, has been transfected into a variety of cells, with the outcome that the haplotype containing T variants at 3425, 2677, and 1236 had some impaired functions (37). In this study, the polymorphisms were not associated with altered message and protein levels but were likely associated with the timing of cotranslational folding and membrane insertion. The authors of this study measured surface Pgp with MRK16 antibody such that we can make a direct comparison between the levels of Pgp seen in their system and the Pgp seen in AML blasts. We gave details of 75th centile Pgp in AML samples in Table 4. On the same scale, the transfected Pgp in Kimchi-Sarfaty's system has a peak value at least 2 orders of magnitude higher. The functional effects seen in their system were dependent on inserted DNA concentration, which was hypothesized by the authors to be attributable to depletion of rare codon-specific tRNAs. We therefore very much doubt that these effects would be applicable to primary AML samples. Nevertheless, we have not excluded the possibility that other polymorphisms combine with C3435T to induce an effect in AML samples.

In contrast to the protein results, we found no significant effect of *ABCB1* polymorphisms on retention of the Pgp substrate rhodamine 123 in the presence of PSC-833. Discrepancies between protein and functional results have frequently been observed in AML [our results and those of others (38)], and the effect of the polymorphic *ABCB1* 3435TT variant on Pgp protein expression was overridden by other factors when it came to functional rhodamine efflux (Fig. 1). Several protein phosphatases and kinases are associated with Pgp function (39, 40) and could account for this effect. Drug-resistant cells are known to have different patterns of phosphatase and kinase activity compared with their drug-sensitive parent cells (41). However, although the effect of genetic polymorphism at the protein level is lost at the level of xenobiotic efflux, it should be borne in mind that Pgp has a drug-independent role in resistance to apoptosis, associated with modulation of ceramide and fas pathways (42–44). There have been no investigations of whether polymorphic variants affect such antiapoptotic functions.

This study has provided no evidence supporting the possibility of a role for *ABCB1* polymorphisms in the pathogenesis of AML. An age or cytogenetics bias in polymorphic subgroups or a lack of Hardy-Weinberg equilibrium in genotype distribution might suggest the possibility that bone marrow cells with a particular *ABCB1* genotype have an increased likelihood of transformation, but our samples showed no abnormal distributions.

In summary, we have shown that Pgp expression and function in AML are strongly affected by acquired, leukemia-specific factors. In addition, we have shown that the genetic polymorphisms of *ABCB1* at positions 2677 and 3435 have a significant effect on Pgp protein expression, but our data indicate that only if Pgp is turned on by leukemia-specific factors do genetic factors secondarily affect expression levels.

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References

1. Gottesman MM, Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. FEBS Lett 2006;580:998–1009.
2. Leith C. Multidrug resistance in leukemia. Curr Opin Hematol 1998;5:287–91.
3. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219–34.
4. Pallis M, Turzanski J, Higashi Y, Russell N. P-glycoprotein in acute myeloid leukaemia: therapeutic implications of its association with both a multidrug-resistant and an apoptosis-resistant phenotype. Leuk Lymphoma 2002;43:1221–8.
5. Labialle S, Gayet L, Marthinet E, Rigal D, Baggetto LG. Transcriptional regulators of the human multidrug resistance 1 gene: recent views. Biochem Pharmacol 2002;64:943–8.
6. Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo*. Proc Natl Acad Sci U S A 2000;97:3473–8.
7. Kim RB, Leake BF, Choo EF, et al. Identification of functionally variant MDR1 alleles among European Americans and African Americans. Clin Pharmacol Ther 2001;70:189–99.
8. Moriya Y, Nakamura T, Horinouchi M, et al. Effects of polymorphisms of MDR1, MRP1, and MRP2 genes on their mRNA expression levels in duodenal enterocytes

of healthy Japanese subjects. Biol Pharm Bull 2002; 25:1356–9.

9. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM. P-glycoprotein: from genomics to mechanism. Oncogene 2003;22:7468–85.

10. Woodahl EL, Yang Z, Bui T, Shen DD, Ho RJ. MDR1 G1199A polymorphism alters efflux transport activity of P-glycoprotein. J Pharmacol Exp Ther 2004;310: 1199–207.

11. Kimchi-Sarfaty C, Gribar JJ, Gottesman MM. Functional characterization of coding polymorphisms in the human MDR1 gene using a vaccinia virus expression system. Mol Pharmacol 2002;62:1–6.

12. Morita N, Yasumori T, Nakayama K. Human MDR1 polymorphism: G2677T/A and C3435T have no effect on MDR1 transport activities. Biochem Pharmacol 2003;65:1843–52.

13. Illmer T, Schuler US, Thiede C, et al. MDR1 gene polymorphisms affect therapy outcome in acute myeloid leukemia patients. Cancer Res 2002;62:4955–62.

14. Cascorbi I, Gerloff T, Johne A, et al. Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. Clin Pharmacol Ther 2001;69:169–74.

15. Pallis M, Das-Gupta E. Flow cytometric measurement of functional and phenotypic p-glycoprotein. In: Blumenthal RD, editor. Chemosensitivity. Totowa: Humana Press; 2005.

16. Borowitz MJ, Guenther KL, Shults KE, Steltzer GT. Immunophenotyping of acute leukemia by flow cytometric analysis: use of CD45 and right angle light scatter to gate on leukemic blasts in three-color analysis. Am J Clin Pathol 1993;100:534–40.

17. Rainer RO, Hodges L, Seltzer GT. CD 45 gating correlates with bone marrow differential. Cytometry 1995;22:139–45.

18. Broxterman HJ, Sonneveld P, Feller N, et al. Quality control of multidrug resistance assays in adult acute leukaemia: correlation between assays for P-glycoprotein expression and activity. Blood 1996;87:4809–16.

19. Pallis M, Turzanski J, Harrison G, et al. Use of standardised flow cytometric determinants of multidrug resistance to analyse response to remission induction chemotherapy in patients with acute myeloblastic leukaemia. Br J Haematol 1999;104:307–12.

20. Pallis M, Turzanski J, Langabeer S, Russell N. Reproducible flow cytometric methodology for measuring multidrug resistance in leaukaemic blasts. In: Kaspers G, Pieters R, Veerman A, editors. Rec Adv Exp Biol. New York: Kluwer Academic; 1999. p. 77–88.

21. Pepper C, Bentley P, Hoy T. Regulation of clinical chemoresistance by bcl-2 and bax oncoproteins in B-cell chronic lymphocytic leukaemia. Br J Haematol 1996;95:513–7.

22. van den Heuvel-Eibrink MM, Wiemer EA, de Boevere MJ, et al. MDR1 gene-related clonal selection and P-glycoprotein function and expression in relapsed or refractory acute myeloid leukemia. Blood 2001;97:3605–11.

23. Pallisgaard N, Clausen N, Schroder H, Hokland P. Rapid and sensitive minimal residual disease detection in acute leukemia by quantitative real-time RT-PCR exemplified by t(12;21) TEL-AML1 fusion transcript. Genes Chromosomes Cancer 1999;26:355–65.

24. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood 1998;92: 2322–33.

25. Harrison CJ, Martineau M, Secker-Walker LM. The Leukaemia Research Fund/United Kingdom Cancer Cytogenetics Group Karyotype Database in acute lymphoblastic leukaemia: a valuable resource for patient management. Br J Haematol 2001;113:3–10.

26. Beck WT, Grogan TM, Willman CL, et al. Methods to detect p-glycoprotein-associated multidrug-resistance in patients tumors: consensus recommendations. Cancer Res 1996;56:3010–20.

27. Marie JP, Huet S, Faussat AM, et al. Multicentric evaluation of the MDR phenotype in leukemia. Leukemia 1997;11:1086–94.

28. Pallis M, Fisher J, Truran L, Grundy M, Russell N, Burnett A. Reproducible measurements of AML blast p-glycoprotein function in two centre analysis. Blood 2005;105:1367–8.

29. Hunault M, Zhou D, Delmer A, et al. Multidrug resistance gene expression in acute myeloid leukemia: major prognosis significance for *in vivo* drug resistance to induction treatment. Ann Hematol 1997;74: 65–71.

30. Willman CL. Immunophenotyping and cytogenetics in older adults with acute myeloid leukemia: significance of expression of the multidrug resistance gene-1 (MDR1). Leukemia 1996;10 Suppl 1: S33–5.

31. Schaich M, Harbich-Brutscher E, Pascheberg U, et al. Association of specific cytogenetic aberrations with mdr1 gene expression in adult myeloid leukemia and its implication in treatment outcome. Haematologica 2002;87:455–64.

32. van den Heuvel-Eibrink MM, Wiemer EA, de Boevere MJ, et al. MDR1 expression in poor-risk acute myeloid leukemia with partial or complete monosomy 7. Leukemia 2001;15:398–405.

33. van der Holt B, Van den Heuvel-Eibrink MM, Van Schaik RH, et al. ABCB1 gene polymorphisms are not associated with treatment outcome in elderly acute myeloid leukemia patients. Clin Pharmacol Ther 2006; 80:427–39.

34. Pallis M, Turzanski J, Grundy M, Seedhouse C, Russell N. Resistance to spontaneous apoptosis in acute myeloid leukaemia blasts is associated with p-glycoprotein expression and function, but not with the presence of FLT3 internal tandem duplications. Br J Haematol 2003;120:1009–16.

35. Tafuri A, Andreeff M. Kinetic rationale for cytokine-induced recruitment of myeloblastic leukemia followed by cycle-specific chemotherapy *in vitro*. Leukemia 1990;4:826–34.

36. Wang D, Johnson AD, Papp AC, Kroetz DL, Sadee W. Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>Taffects mRNA stability. Pharmacogenet Genomics 2005;15:693–704.

37. Kimchi-Sarfaty C, Oh JM, Kim IW, et al. A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 2007;315:525–8.

38. Leith CP, Chen IM, Kopecky KJ, et al. Correlation of multidrug resistance (MDR-1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: identification of discordant MDR-/efflux+ and MDR1+/efflux- cases. Blood 1995;86:2329–42.

39. Chambers TC, Zheng B, Kuo JF. Regulation by phorbol ester and protein kinase C inhibitors, and by a protein phosphatase inhibitor (okadaic acid), of P-glycoprotein phosphorylation and relationship to drug accumulation in multidrug-resistant human KB cells. Mol Pharmacol 1992;41:1008–15.

40. Rumsby MG, Drew L, Warr JR. Protein kinases and multidrug resistance. Cytotechnology 1998;27: 203–24.

41. Ratnasinghe D, Phang JM, Yeh GC. Differential expression and activity of phosphatases and protein kinases in adriamycin sensitive and resistant human breast cancer MCF-7 cells. Int J Oncol 1998;13:79–84.

42. Pallis M, Russell N. P-glycoprotein plays a drug-efflux-independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. Blood 2000;95:2897–904.

43. Turzanski J, Grundy M, Shang S, Russell N, Pallis M. 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 2005;33:62–72.

44. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci U S A 1998;95:7024–9.