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A potential role of fetal hemoglobin in the development of multidrug resistance

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ABSTRACT

Our previous data from a human leukemic cell line made resistant to the nucleoside analog (NA) 9-β-*D*-arabinofuranosylguanine (AraG) revealed a massive upregulation of fetal hemoglobin (HbF) genes and the ABCB1 gene coding for the multidrug resistance P-glycoprotein (P-gp). The expression of these genes is regulated through the same mechanisms, with activation of the p38-MAPK pathway and inhibition of methylation making transcription factors more accessible to activate these genes. We could show that AraG, as well as other NAs, and P-gp substrates could induce global DNA demethylation and induction of Hbγ and P-gp both at the mRNA and protein expression level. We speculate that the expression of HbF prior to drug exposure or in drug-resistant cell lines is a strategy of the cancer to gain more oxygen, and thereby survival benefits. We also believe that P-gp may be induced in order to excrete Hb degradation products from the cells that would otherwise be toxic. By using Hbγ siRNA and pharmacological inhibitors of HbF production we here present a possible relationship between HbF induction and multi-drug resistance in a human leukemia cell line model.

1. Introduction

The mechanisms involved in the development of resistance to anti-cancer treatment have long been of our interest. We have in previous work shown that the nucleoside analog (NA) 9-β-D-arabinofuranosylguanine (AraG), the demethylated substance of Nelarabine (Arranon®), used to treat different leukemic disorders, induces global DNA demethylation in human leukemia cell lines [1], accompanied by a massive induction of fetal hemoglobin (HbF) genes, and the multidrug-resistance P-glycoprotein (P-gp) pump [1]. Recent immunohistochemical experiments also showed the induction of HbF synthesis in leukemia cells, as well as in the progenitors of red blood cells of untreated leukemia patients [2]. Such Hb levels may be a measurement of the degree of DNA demethylation, since the β-globin locus on chromosome 11p15.5, housing the different fetal hemoglobin genes, is especially sensitive to demethylation [3,4]. It is also well known that several cytotoxic compounds used e.g. as chemotherapy can induce the expression of HbF [5–10]. The β-globin locus and the ABCB1 gene coding for P-gp can be induced and regulated through the p38-MAPK intracellular signaling pathway along with demethylation by irreversible inhibition of DNA methyl transferases [11], and activation of c-jun/AP-1 transcription factors [12–14]. P-gp expression is also of prognostic significance in different leukemia disorders, and the multidrug-resistance phenotype usually develops during treatment [15,16] as a consequence of induction rather than selection of P-gp expressing cells [17].

We also believe that expression of HbF in cancer prior to treatment may be a strategy of the cancer to gain increased access of oxygen, since HbF have a higher affinity for oxygen than the adult hemoglobin, and therefore we assume that HbF is a potential marker of malignancy. It may also be a consequence of surrounding environmental factors able to induce global DNA demethylation and activation of normally silent genes. This is supported by evidence noting that DNA hypomethylation as well as DNA hypermethylation are involved in promoting cancer [18].

Hbγ is the most prominent subunit present during fetal life, and therefore this gene was investigated together with the ABCB1 gene, coding for P-gp, to see whether they could be functionally connected by using a cell line expressing high and one expressing low or no amounts of HbF and P-gp. Our speculations regarded the possibility that not all of the P-gp induction during chemotherapy treatment is a direct response of the cell to excrete the drugs, but may be a consequence of the excess hemoglobin produced in the cells. Here we present data that the expressions of HbF genes and the ABCB1 gene seem to be closely regulated possibly in order for the cells to excrete excess hemoglobin degradation products. We also believe that this knowledge may be of importance in cancer chemotherapy treatment and that preventing HbF gene induction could possibly be able to make patients more susceptible to drugs and avoid, to some extent, the development of multi-drug resistance.

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2. Materials and methods

2.1. Reagents and drugs

9-β-*D*-arabinofuranosylguanine (AraG, Arranon®) was obtained from R.I. Chemical (Orange, CA, USA), daunorubicin (Dnr, Cerubidin®), an anthracycline and known P-gp substrate was from Rhône-Poulenc Rorer (Bristol, UK). Cytarabine (AraC, Cytosar®), a nucleoside analog with demethylating activity was from Pharmacia & Upjohn (Stockholm, Sweden), cyclosporin A (Sandiummun®), a well characterized and first generation inhibitor of P-gp function was from Novartis (Stockholm, Sweden), laniquidar, a third generation inhibitor of P-gp was from Johnson & Johnson (Schaffhausen, Switzerland), and 5-AZA, a demethylating nucleoside analog, and SB203580 were from Sigma Aldrich (Stockholm, Sweden). The specific p38 kinase inhibitor SB203580, a pyridinyl imidazole, can specifically inhibit the induction of HbF genes from the β-globin locus [19] as well as the ABCB1 gene [20]. Western blot equipment and reagents were from Bio-Rad Laboratories (Hercules, CA, USA), cell-culturing reagents were from Gibco, Life Technologies (Paisley, UK), real-time PCR equipment and reagents were from Applied Biosystems (Foster City, CA, USA), and additional chemicals were purchased from Sigma Aldrich.

2.2. Cell lines and culturing

Cell lines used were MOLT-4, a T-cell acute lymphoblastic leukemia cell line (American Type Culture Collection, ATCC) and its variant MOLT-4/AraG made resistant to AraG (0.9 μM). They were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C with 5% CO_2 in a humidified atmosphere and were subcultured twice weekly.

2.3. Sample preparation, PCR, and western blotting

RNA was extracted with the RNeasy Mini Kit (Qiagen, Solna, Sweden) and complementary DNA was produced using the High Capacity Archive kit (Applied Biosystems). Quantification of hemoglobin γ (Hs00361131_g1) and ABCB1 (Hs00184500_m1) expressions were performed using Taqman® Gene Expression Assays (20X), with the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene (Hs00266705_g1) as housekeeping gene as previously described [1].

Total cellular proteins were extracted from cells as previously described [21], and Hbγ and P-gp were visualized using western blotting. Criterion Precast 4–15% Tris-HCl gels were loaded with 10 μg of protein and run in a sodium dodecyl sulfate (SDS) running buffer (0.4 M glycine, 50 mM Tris-HCl, and 14 mM SDS) at 100 V for 2 h. The proteins were transferred onto an Immune Blot PVDF membrane in a transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) at 50 V for 1 h. Mouse anti-hemoglobin γ (1:200) antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and mouse anti-P-gp antibody and rabbit anti- β-actin antibody (1:200 and 1:1000, Abcam, Cambridge, UK) were used. All secondary peroxidase-labeled antibodies (1:1000) were from Amersham Biosciences (Little Chalfont, UK). The blots were developed using Supersignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

2.4. MTT-assay

The drug cytotoxicity assay was performed using methyl thiazole tetrazolium (MTT) as previously described [21], and was used to determine the half maximal inhibitory concentration values (IC_{50}-values) of the different drugs and inhibitors.

2.5. DNA pulse-labeling and global DNA methylation

Cells were incubated with different drugs at 0.5×10^6 cells/ml for different periods of time, and then pulse-labeled to measure the global methylation status. Cells were washed in pre-heated PBS (37 °C) in order to rinse out all traces of drugs and then resuspended in pulse-labeling media containing 1.5 mmol/l L-[methyl-^{3}H]methionine (37 MBq, specific activity 75.6 Ci/mmol, Perkin Elmer, Waltham, MA, USA) as methyl donor, and 0.2 μmol/l [methyl-^{14}C]thymidine (1.85 MBq, specific activity 61 mCi/mmol, Amersham Biosciences, Little Chalfont, UK) to be incorporated into the newly synthesised DNA. DNA was then extracted with QIAamp DNA mini kit (Qiagen) according to the manufactureŕs recommendations after 4 h of incubation. A ratio between ^{3}H and ^{14}C was then used to estimate methylation and the results were compared to the results from cells cultured without drug.

2.6. SiRNA transfection

Transfection of Hbγ small interfering RNA (Santa Cruz) was done using electroporation according to the protocol previously described [22]. Cells were electroporated with 250 nM Hbγ siRNA initially, and then after 24, 72, and 336 h (two weeks). Hbγ and ABCB1/P-gp mRNA and protein expression was then evaluated using real-time PCR and western blotting.

2.7. Inhibitor and drug exposure experiments

Cells were cultured with AraG or Dnr with or without CsA, laniquidar, or SB203580. The inhibitors were added 2 h before the addition of AraG or Dnr (recommendations from suppliers). Cells were then harvested and assayed for Hbγ and ABCB1 mRNA and/or protein expression. MTT assays were performed on the cells after all traces of drug had been washed away using dilutions of AraG or Dnr in triplicates.

2.8. Flow cytometry

In order to determine the function of drug transportation by the P-gp pump, cells which had been incubated with drugs and/or P-gp inhibitors or SB203580 were loaded with rhodamine 123 (5.2 μM), a P-gp substrate, at 37 °C for 30 min. Cells were then centrifuged at 600 × g and resuspended in PBS buffer to a concentration of 2×10^6 cells/ml, and movement of rhodamine 123 from the cells was analyzed using a FACS Calibur at 20, 50 and 120 min after rhodamine 123 loading. Data were further processed using Cellquest (Becton-Dickinson, San Jose, CA).

3. Results

3.1. MTT, and basal expression of Hbγ and ABCB1

The IC_{50}-values of 5-AZA, AraC, AraG, and Dnr, as well as CsA and laniquidar were determined using the MTT-assay. MOLT-4/AraG was highly insensitive to AraG as expected and cross-resistant to several other drugs (Table 1). MOLT-4/AraG was also the only cell line with significant Hbγ and ABCB1/P-gp expression (Table 1).

3.2. DNA demethylation and Hbγ/ABCB1 induction

In order to investigate whether the above drugs were able to demethylate DNA and induce Hbγ and ABCB1 expression,

Table 1
IC_{50}-values of drugs and basal mRNA expression of HbF and ABCB1.

	MOLT-4	MOLT-4/AraG
5-AZA (μM)	4.2 ± 0.6	6.5 ± 0.2
Dnr (μM)	0.04 ± 0.001	9.2 ± 1.1
AraC (μM)	0.05 ± 0.001	2.9 ± 0.3
AraG (μM)	1.9 ± 0.05	276.0 ± 14.2
CsA (μM)	1.9 ± 0.01	13.2 ± 0.9
Laniquidar (μM)	5.0 ± 0,08	7.0 ± 0.4
Hbγ	0.000002	0.95
ABCB1 expression	n.d.	1.02

The sensitivity for different drugs was tested in two cell lines using the MTT-assay and was expressed as the half maximal inhibitory concentration (IC_{50}). The mRNA expression of Hbγ and ABCB1 is also shown (as mean value from two different measurements) and was assessed using real-time PCR and expressed as a qutoient between Hbγ and the house-keeping gene GAPDH.

MOLT-4 cells, with low or absent expression of Hbγ and ABCB1, were incubated with these drugs at concentrations close to their IC_{50}-values. At 4 h and 72 h after adding drugs there were some demethylation and induction of Hbγ and to some extent ABCB1 (Table 2). Hbγ was induced early in the cells, while ABCB1 was induced later, if induced at all during the 72 h incubation (Table 2). At 72 h, DNA demethylation was induced for all drugs tested, and was most prominent for laniquidar and 5-AZA.

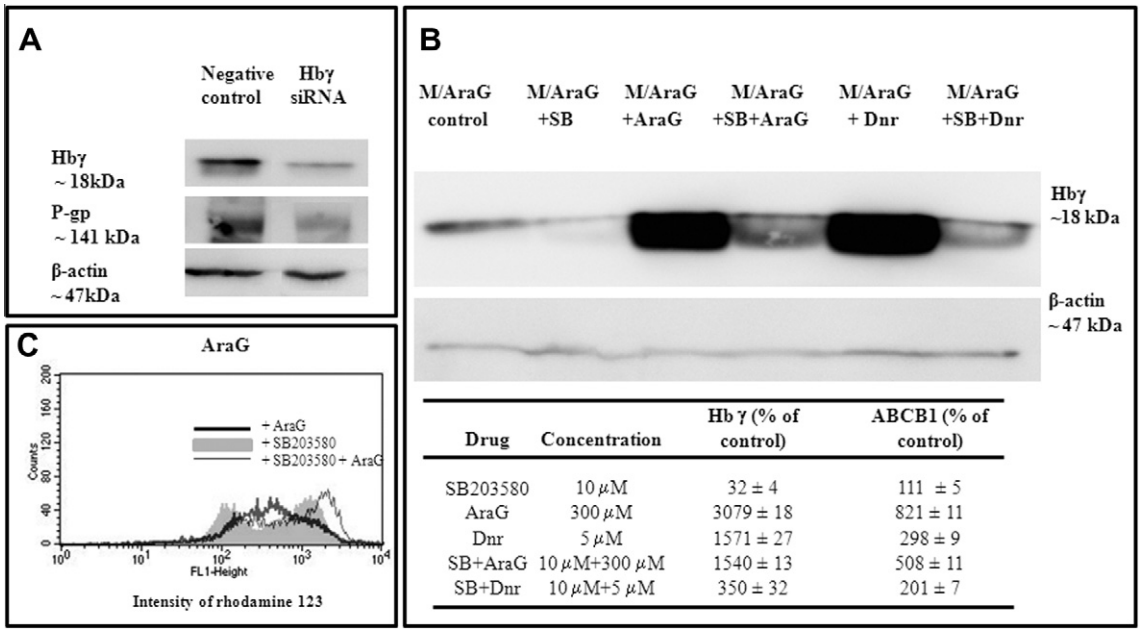
3.3. Hbγ siRNA in MOLT-4/AraG cells

To show a possible functional relationship between HbF and ABCB1, siRNA against Hbγ was used in the MOLT-4/AraG cells in order to suppress its expression. It took several transfections over several days in order for Hbγ to decrease its expression with 70%, which also led to reduced ABCB1 expression in MOLT-4/AraG cells at 480 h or 20 days after the first transfection (approximately 30%). At the protein expression level, both Hbγ and P-gp were decreased at this time-point after transfection (Fig. 1(A)), but the decrease did however not lead to any significant increased drug sensitivity in the MOLT-4/AraG cells tested both after 96 h and 20 days after initial transfection (not shown).

Table 2
Induction of HbF and ABCB1 after global demehtylation.

4 h	ABCB1 expression	Hbγ expression	Global methylation % of control	72 h	ABCB1 expression	Hbγ expression	Global methylation % of control
5AZA 5 uM	n.d.	5.9	95	5AZA 5 uM	n.d.	93	64
Dnr 0.1 uM	n.d.	9.5	96	Dnr 0.1 uM	n.d.	27	63
AraC 0.1 uM	n.d.	5.5	109	AraC 0.1 uM	0.2	137	93
AraG 2 uM	n.d.	6.5	105	AraG 2 uM	0.07	113	77
CsA 2 uM	3.4	23.3	96	CsA 2 uM	4.0	65	86
Laniq 5 uM	0.2	6.9	91	Laniq 5 uM	0.02	172	60

The human leukemic MOLT-4 cell line was treated with different drugs, cells were harvested at different time-points and DNA, RNA, and proteins were extracted. The mRNA expression (x-fold induction compared to untretated control cells, mean from two different experiments) of Hbγ and ABCB1 was detected with real-time PCR and global DNA was estimated using DNA pulse-labeling (mean from two different experiments). Protein expression using western blotting was unsuccessful due to low protein expression. n.d.-not detectable.



Drug	Concentration	Hb γ (% of control)	ABCB1 (% of control)
SB203580	10 μM	32 ± 4	111 ± 5
AraG	300 μM	3079 ± 18	821 ± 11
Dnr	5 μM	1571 ± 27	298 ± 9
SB+AraG	10 μM+300 μM	1540 ± 13	508 ± 11
SB+Dnr	10 μM+5 μM	350 ± 32	201 ± 7

Fig. 1. (A) Nucleoside analog resistant MOLT-4/AraG cells expressing high levels of Hbγ and ABCB1/P-gp were treated with Hbγ siRNA several times during several days in order to decrease its expression. Hbγ and ABCB1 protein expression was reduced after 20 days compared to cells transfected with a negative control siRNA and compared to β-actin. (B) MOLT-4/AraG cells treated with AraG or Dnr alone or in combination with SB203580 during 5 days were subjected to western blotting and real-time PCR measurement of Hbγ and ABCB1/P-gp and compared to untreated cells. Cells treated with AraG or Dnr alone showed increased Hbγ and ABCB1 expression while incubation or co-incubation with SB203580 reduced their expressions. (C) The same cells were loaded with the P-gp substrate dye rhodamine 123. Efflux of this dye was determined using flow cytometry at different time-points and here the data at 120 min after loading with rhodamine 123 are shown using SB203580 in combination with AraG. Curves shifting right-more rhodamine 123 retained intracellularly, and therefore decreased P-gp function.

Table 3
Incubation with cytotoxic drugs and SB203580.

Treatment	MTT data
MOLT-4	AraG IC50 (μM)
Untreated	2 ± 0.01
AraG 1 μM	10 ± 2
SB203580 10 μM + AraG 1 μM	2 ± 0.05
MOLT-4/AraG	AraG IC50 (μM)
Untreated	276 ± 19
AraG 300 μM	959 ± 32
SB203580 10 μM + AraG 300 μM	786 ± 28
MOLT-4/AraG	Dnr IC50 (μM)
Untreated	11 ± 2
Dnr 5 μM	460 ± 12
SB203580 10 μM + Dnr 5 μM	28 ± 4

MOLT-4 cells or MOLT-4/AraG cells were treated for five days with different concentrations of AraG, Dnr, or SB203580 combined with AraG or Dnr. Cells were then washed thouroughly and MTT assay was performed with the cells using dilutions of AraG or Dnr for 72 h to see whether resistance to these drugs had developed.

3.4. SB203580 inhibition of HbF and ABCB1

Another approach was tested in order to decrease the expression of HbF. Incubating the drug-reistant MOLT-4/AraG cells with increasing concentrations of SB203580 (5–15 μM) during five days made the cells dose-dependently more sensitive to Dnr and AraG in a following MTT-assay (not shown). Therefore MOLT-4 and MOLT-4/AraG cells were incubated with AraG (1 or 300 μM respectively), Dnr (5 μM) or a combination of SB203580 (10 μM) and AraG or Dnr for five days. Drugs were washed away and MTT assay was performed with the cells using drug dilutions of AraG or Dnr. Incubating cells with SB203580 together with AraG or Dnr increased the sensitivity to these drugs (Table 3) compared to untreated cells. MOLT-4 cells did not develop any significant resistance to Dnr during this time but SB203580 had a substantial effect of reversing resistance to Dnr in the MOLT-4/AraG cells (Table 3). As depicted in Fig. 1(B), both AraG and Dnr alone increased Hbγ mRNA and protein expression as well as ABCB1 mRNA expression during five days incubation in the MOLT-4/AraG cells. In the samples where SB203580 had been added alone or in combination with AraG and Dnr, Hbγ mRNA and protein expression, and ABCB1 expression were decreased compared to cells incubated with AraG or Dnr alone (Fig. 1(B)). SB203580 did not show any demethylating capacity of DNA as measured with DNA pulse-labeling (not shown).

3.5. Flow cytometry to determine P-gp function

Since mRNA expression do not necessarily reflect protein expression, we decided to look at P-gp function using a functional P-gp assay with transport of the rhodamine 123 dye. Incubating MOLT-4/AraG cells together with SB203580 (Fig. 1(C)) for five days shifted the histogram curve to the right illustrating a decreased P-gp function and more of the rhodamine retained intracellular as compared to cells incubated with AraG only. We could not detect a significant shift in cells incubated with Dnr (not shown). The same trend was seen with cells incubated with CsA or laniquidar, and AraG as well (not shown). Cells incubated with drugs during 24 h were also included in the analysis but without any detectable differences (not shown).

4. Discussion

Previous findings from a leukemic cell line made resistant to the NA AraG, showed a massive upregulation of the multidrug resistance ABCB1 gene and different HbF genes, as well as a global demethylating effect of this drug on DNA. We examined the possibility that the P-gp pump may be induced along with the HbF genes in order to help with excretion of hemoglobin degradation products, and thereby P-gp would be induced, not only in response to P-gp substrate drugs but to demethylation and induction of HbF as well. P-gp generates resistance to the widest variety of cytotoxic drugs [23] and its regulation at the gene level is highly complex, probably involving different regulatory mechanisms in malignant cells and in normal tissue [20]. We believe that the HbF genes and the ABCB1 gene are sensitive to the same transcriptional activation, because the P-gp pump is aiding in hemoglobin excretion. HbF genes are normally silenced shortly after birth and make up less than 1% of the hemoglobin content in healthy non-anemic individuals [24]. However, these genes may be reactivated under malignant conditions, and HbF containing blood cells may be present in both solid tumors and in leukemia [20,25].

In this study we induced ABCB1 and Hbγ gene expression and global DNA demethylation with a variety of cytotoxic drugs in the human leukemic MOLT-4 cell line because of its more or less absent expression of ABCB1 and Hbγ. We also used its AraG-resistant variant MOLT-4/AraG expressing high levels of these genes and its protein products.

In order to study the interaction between P-gp and Hbγ, we used two different P-gp inhibitors, the first generation inhibitor CsA and the third generation inhibitor laniquidar as well as the p38-MAPK inhibitor SB203580, known to suppress the transcription factor AP-1, common to both fetal hemoglobin genes and the ABCB1 gene [14,20]. All of the drugs with demethylating capacity induced the expression of Hbγ, and to some extent ABCB1, in the drug-sensitive MOLT-4 cells after 72 h incubation, and the Hbγ induction was present prior to ABCB1 if present at all during this time of incubation. The P-gp inhibitors also induced the expression of Hbγ and ABCB1, and showed a demethylating effect on DNA, which may seem a bit conflicting since they also inhibit this pump. Many P-gp modulators/inhibitors act as P-gp substrates and function as competitive inhibitors preventing the outward efflux of cytotoxic drugs [26]. If the high HbF levels remains in the cells, they will still need P-gp in order to excrete these compounds and one strategy for the cell could then be to increase the expression of this pump in order to compensate for its inhibition.

We attempted to silence Hbγ in the HbF overexpressing MOLT-4/AraG cell line using siRNA. This was difficult probably due to the massive overproduction of HbF and because of the fact that other HbF subunits besides Hbγ were activated in the cells, as we have previously shown [1]. Even though mRNA and protein measurements showed reduced Hbγ and ABCB1/P-gp levels, the cells did not display any significant altered sensitivity to AraG or Dnr. Instead, since the reduction was not enough to affect the cytotoxicity we used a pharmacological approach to inhibit the induction of HbF genes, and the ABCB1 gene using the pyridinyl imidazole inhibitor SB203580 which specifically inhibits p38-MAPK [27]. SB203580 affected drug toxicity making the MOLT-4/AraG cells more sensitive to AraG and Dnr and prevented the induction of resistance towards AraG in drug-sensitive MOLT-4 cells incubated with AraG. SB203580 used together with drugs markedly decreased the Hbγ protein expression and also reduced its Hbγ and ABCB1 mRNA expression. Flow cytometry analysis of these cells using the P-gp substrate rhodamine 123 showed some increased retention of this dye in cells treated with SB203580, CsA, and laniquidar alone or in combination with AraG or to some extent Dnr. Even though CsA, laniquidar, and SB203580 all have the capacity to reverse P-gp mediated drug resistance their mechanisms of action differ considerably. CsA and laniquidar apparently demethylate DNA and induces HbF expression, and ABCB1 expression probably by acting at the MAPK-pathway, but inhibits the transport of drugs by serving as substrates of the pump [28]. Activation of MAPK and

p38 is however dependent of both cell type and stimulus and may e.g. induce cell death in some cells while promoting survival in others, and may in some cells be linked even to tumorigenesis [29]. SB203580 does not induce demethylation or HbF/ABCB1 induction, but still reverse the effect of P-gp. The results indicate that ABCB1/P-gp expression is partly decreased as a response to the decreased HbF levels when SB203580 is used, and that these genes are somehow closely regulated so that elevated P-gp expression follows elevated HbF levels possibly as a protective mechanism for the cell. In order to really test our hypothesis we would need a specific inhibitor of the HbF genes without acting on the ABCB1 expression or a more effective siRNA method. Using siRNA gave indications that decreasing the Hbγ also reduced ABCB1/P-gp. In patients treated with CsA, at levels that partially restore the drug sensitivity, severe side-effects have shown to be hyperbilirubinemia [30] perhaps as a consequence of the decreased P-gp transportation capacity of hemoglobin degradation products. Bilirubin has a low but significant affinity for P-gp [31] and we believe that the strategy of using SB203580 or similar molecules may be a safer way of modulating P-gp expression without subsequent hyperbilirubinemia and possibly other toxic side effects.

Not only cytotoxic drugs have the capacity to activate p38-MAPK and demethylation but also UV light, heat, osmotic shock, inflammatory cytokines, and growth factors [29]. This is probably the reason why high HbF expression can be seen in samples from patients prior to treatment. All this evidence imposes the involvement of HbF expression, possibly in the malignant process, and clearly in the development of drug resistance. These findings may confer important therapeutic and prognostic implications in the treatment of leukemia and maybe other tumors in the future.

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