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ORIGINAL ARTICLE

Induction of fetal hemoglobin and ABCB1 gene expression in 9-*β*-*D*-arabinofuranosylguanine-resistant MOLT-4 cells

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Abstract
Purpose To characterize resistance mechanisms to the nucleoside analog 9-*β*-*D*-arabinofuranosylguanine (AraG) in the T-cell acute lymphoblastic leukemia cell line MOLT-4 and its AraG-resistant variant.
Methods A gene expression microarray analysis was performed, as well as gene expression and enzyme activity measurements of key enzymes in the activation of AraG. Cytotoxicity of AraG and cross-resistance to other compounds were evaluated using a standard cytotoxicity assay.
Results Gene expression microarray analysis revealed that fetal hemoglobin genes and the multidrug resistance ABCB1 gene, encoding the drug efflux pump P-gp, were the most highly upregulated genes in the resistant cells, while genes traditionally associated with nucleoside analog resistance were not. Fetal hemoglobin and ABCB1 induction can be due to global DNA hypomethylation. This phenomenon was studied using AraG during a period of 4 weeks in MOLT-4 cells and the lung adenocarcinoma cell line A549, leading to up-regulation of hemoglobin gamma and ABCB1 as well as DNA hypomethylation. Inhibiting P-gp in the AraG-resistant MOLT-4 cells led to decreased proliferation, reduced hemoglobin expression, and highly induced ABCB1 expression.
Conclusions We show that AraG can cause hypomethylation of DNA and induce the expression of the fetal hemoglobin gamma gene and the ABCB1 gene. We speculate that the induction of ABCB1/P-gp may occur in order to help with excretion of hemoglobin degradation products that would otherwise be toxic to the cells, and we present data supporting our theory that P-gp may be linked to the induction of hemoglobin.

Keywords 9-*β*-D-arabinofuranosylguanine · Fetal hemoglobin · P-glycoprotein · Microarray · Hypomethylation

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Introduction

The antileukemic drug 9-*β*-D-arabinofuranosylguanine (AraG) was synthesized in the 1950s but only recently brought into clinical use for the treatment of relapsed or refractory T-cell acute lymphoblastic leukemia (T-ALL) or lymphoblastic lymphoma [1–3]. The drug is used clinically as a prodrug, nelarabine (Atriance®) which is more water soluble than AraG and is demethylated in blood to AraG [2]. AraG is then activated intracellularly by the cytosolic enzyme deoxycytidine kinase (dCK) and the mitochondrial enzyme deoxyguanosine kinase [4, 5]. The high content of mitochondria in neurons can partially explain why severe side effects of AraG treatment include life-threatening neurotoxicity [1]. Inherited or acquired resistance to nucleoside analog treatment is a common phenomenon leading to treatment failure. In order to study possible resistance mechanisms to AraG, we performed a gene expression

microarray analysis of the human leukemic T-ALL cell line MOLT-4 and its AraG-resistant variant.

In this study, we show that traditional nucleoside analog resistance genes were only moderately affected in the resistant MOLT-4/AraG cells compared with parental cells. However, we could see a massive induction of fetal hemoglobin genes and the multidrug resistance gene ABCB1 coding for the drug efflux pump P-glycoprotein (P-gp). We suggest that AraG induces the expression of hemoglobin, ABCB1, and other genes by hypomethylation of DNA and that P-gp may be induced to help excrete fetal hemoglobin waste products from the cells since AraG is not considered a substrate of this pump.

Materials and methods

Reagents and drugs

9-*β*-*D*-arabinofuranosylguanine (AraG) was obtained from R.I. Chemical (Orange, CA, USA), 6-mercaptopurine (6-MP, Puri-Nethol®) was from GlaxoSmithKline (Solna, Sweden) 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (clofarabine, CAFdA, Evoltra®) was a kind gift from Dr. Howard Cottam, University of California (San Diego, CA. USA), 2-fluoroarabinosyladenine (FaraA) was a gift from Dr. Zéev Shaked, Berlex (Alameda, CA, USA), gemcitabine (dFdC, Gemzar®) was from Eli Lilly (Solna, Sweden), daunorubicin (Dnr, Cerubidin®) was from Rhône-Poulenc Rorer (Bristol, UK), cladribine (CdA, Leustatin®) and laniquidar were from Johnson & Johnson (Schaffhausen, Switzerland), cytarabine (AraC, Cytosar®) was from Pharmacia & Upjohn (Stockholm, Sweden), and cyclosporin A (Sandiummun®) was from Novartis (Stockholm, Sweden). Radioactive substances were from Moravek Biochemicals (Brea, CA. USA), 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 (Stockholm, Sweden).

Cell lines

Cell lines used in this study were the T-cell acute lymphoblastic leukemia cell line MOLT-4 (American Type Culture Collection, ATCC) and variants made resistant to AraG (0.9 μM) or 6-MP (5 μM) as earlier described [4] and the human chronic myelogenous leukemia cell line K562 (ATCC). The human lung adenocarcinoma epithelial cell line A549 was a gift from Dr. S A Coulthard (Newcastle University, Northern Institute for Cancer Research, Newcastle upon Tyne, UK). Cells 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. Cells were kept at 37°C with 5% CO_2 in a humidified atmosphere and were subcultured twice weekly.

Cytotoxicity assay

Sensitivity of the cells to various drugs was determined by the methyl thiazole tetrazolium (MTT) assay as previously described [6]. Triplicate analyses of 20,000 cells per well for MOLT-4 cells and 10,000 cells/well for A549 in 100 μl aliquots in 96-well microtiter plates with 5 μl of drug dilutions were performed. Sensitivity to the different substances, expressed as an IC_{50} value, was calculated as the percent survival compared with control cells.

RNA extraction, reverse transcriptase PCR, and real-time PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Solna, Sweden), and complementary DNA was produced using the High Capacity Archive kit (Applied Biosystems). Details of PCR primers and Taqman probes for dCK and dGK have been published previously [7, 8]. To measure the expression of hemoglobin gamma (Hs00361131_g1) and ABCB1 (Hs00184500_m1), Taqman® Gene Expression Assays (20X) were used, and the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene (Hs00266705_g1) was used as a housekeeping control by dividing the mean quantity of the gene studied with the mean quantity of GAPDH expression after analysis with the Sequence Detection Software 1.3.1 (Applied Biosystems). The reaction mixture contained 1X Taqman Universal Mastermix, forward and reverse primers (800 nM), and a probe (200 nM) or 1X Gene Expression Assay mix. Reaction conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15 s at 95°C and 1 min at 60°C in an ABI Prism 7500 Sequence Detection System.

Protein extraction and enzyme activity measurements

Cells were centrifuged and washed in cold PBS, and the pellets were resuspended in a protein extraction buffer containing 50 mM Tris–HCl (pH 7.6), 4 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.5% Nonidet P40 and then disrupted using liquid nitrogen. After a final 15-min centrifugation at 15,000 ×*g*, the cell supernatants were collected and assayed for dCK and dGK activities.

Enzyme activities were measured by a method published previously based on a modified radiochemical method [6]. Concentrations of the substrates were 10 μM deoxycytidine

([8-^{3}H]-2′-deoxycytidine, specific activity 8.3 Ci/mmol) for dCK and 20 μM 9-*β*-*D*-arabinofuranosylguanine ([8-^{3}H]-guanine-*β*-*D*-arabinofuranoside, specific activity 6.7 Ci/mmol) for dGK. To inhibit the activity of thymidine kinase 2, 1 mM thymidine was added to the dCK reaction and to inhibit the activity of dCK, 50 mM deoxycytidine was added to the dGK reaction. Total radioactivity was measured in a liquid scintillation counter (RackBeta, LKB Wallac, Turku, Finland).

Enzyme activities were expressed as pmol of formed product/min/mg of protein, and the method of Lowry was used to determine protein content in the individual samples using the DC protein assay (Bio-Rad Laboratories).

Western blot

Hemoglobin gamma protein was visualized using western blot. Criterion Precast 4–15% Tris–HCl gels were loaded with 40 μg of protein and run in a sodium dodecyl sulfate (SDS) running buffer containing 0.4 M glycine, 50 mM Tris–HCl, and 14 mM SDS, pH 8.5 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, pH 8.3) at 50 V for 1 h. Mouse antihemoglobin gamma antibody (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and rabbit anti-*β*-actin antibody (1:1,000, Abcam, Cambridge, UK) were used. All secondary peroxidase-labeled antibodies (1:1,000) were from Amersham Biosciences (Little Chalfont, UK). The blots were developed using Supersignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

Microarray analysis

Microarray analyses were performed with RNA from four MOLT-4 samples and four MOLT-4/AraG900 samples using Human Genome Focus Array (Affymetrix, Buckinhamshire, UK) containing more than 8,500 of the most well-characterized human genes. Samples were prepared according to the manufacturer's instructions. Chip data were analyzed using Genespring GX 11.0 (Agilent Technologies, Inc, Santa Clara, CA, USA). Samples were subjected to baseline transformation and thereafter background correction, normalization, and probe summarization were done using the robust multichip averaging (RMA) algorithm [9]. Significantly differentially expressed genes were found using Welch′s *t*-test, followed by the Benjamin-Hochberg post hoc test. Samples were filtered on expression, *P*-value computation was asymptotic, and the cutoff limit was drawn at 0.05. Data were also analyzed by ALMAC Diagnostics (ALMAC Group Ltd, Graigavon, UK) with essentially the same genes found significantly affected.

Global methylation assay

Cells were incubated with AraG (5 or 10 μM) or 6-MP (5 or 10 μM) for 4 weeks (eight passages) and then pulse-labeled to measure the global methylation status. They were maintained at 0.5×10^6 cells/ml for MOLT-4 cells and approximately 70% confluency for A549 cells. Cells were washed in pre-heated PBS (37°C) in order to wash 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 newly synthesized DNA. After 4 h of incubation, the cells were harvested and DNA extracted using the QIAamp® DNA Blood Mini Kit (Qiagen, Solna, Sweden) according to the manufacturer's instructions. 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 with no drug.

Statistical analysis

Additional statistical analyses were performed using the GraphPad Prism, version 4.00, for Windows (GraphPad Software, San Diego, CA, USA). Data were expressed as mean and standard deviation and analyzed using Student′s *t*-test with a *P*-value of ≤ 0.05 being considered significant.

Results

Cytotoxicity of nucleoside analogs and expression and activity of activating enzymes

We have previously described the MOLT-4/AraG900-resistant subclone and compared it to the parental MOLT-4 cell line [4]. We reported then on resistance to AraG but also cross-resistance against other nucleoside analogs and to anthracyclines. Once again, we investigated the pattern of nucleoside analog cytotoxicity and expression and activity of nucleoside analog–activating enzymes in these two cell lines. The AraG-resistant cells showed the highest resistance to AraG, Dnr, and AraC (350-, 1,400-, and 1,500-fold less sensitivity, respectively) compared with the parental cells. They also showed resistance to CdA, CAFdA, and FaraA, although moderately (10- to 70-fold less sensitivity), while the effect of dFdC was slightly increased in the resistant cells as was also shown before by us [4].

The resistant AraG900 subclone had reduced dCK expression and activity (less than half of control cells).

Table 1 Gene regulation assessed by expression microarray analysis

Gene name	Gene ID	Gene description	Gene ontology	Chr	FC
HBZ	NM_00S332	Hemoglobin zeta	Oxygen transport	16p13.3	433
HBG1/2	NM_000184	Hemoglobin gamma	Oxygen transport	11p15.5	411
ABCB/1	NG_011513	P-glycoprotein multidrug resistance	Membrane transporter	7q21.12	225
DLK	NM_003836	Delta-like 1 homolog	Tumor marker	14q32	195
HBA1/2	T50399	Hemoglobin alpha 1/2	Oxygen transport	16p13.3	183
PRAME	NM_206954	Preferentially expressed antigen in melanoma	Inhibits myeloid differentiation	22q11.22	147
MS4A3	NM_001031809	Membrane-spanning 4-domains	Hematopoietic cell specific	11q12.1	137
HBE1	NM_005330	Hemoglobin epsilon 1	Oxygen transport	11pl5.5	136
KLF1	U65404	Kruppel-like factor 1 (erythroid)	Involved in β-and g-globin expression	19p13.13	29.0
BLVRB	NM_000713	Biliverdin reductase B	Hemoglobin degradation	19q13.1	18.8
ABCB6	NM_005689	ATP-binding cassette, subfamily B, member 6	Porphyrin heme synthesis	2q36	3.7
BCL11B	NM_138576	B-cell CLL lymphoma 11B	Hematopoietic cell development	14q32.2	−l2
BCL11A	NM_022893	B-cell CLL lymphoma 11A (zinc finger protein)	Hematopoietic cell development	2p16.1	−9.6
DCK	NM_000788	Deoxycytidine kinase	Nucleotide and nucleic acid metabolism	4q13.3	−4.3
DGUOK	NM_001929	Deoxyguanosine kinase	Nucleotide and nucleic acid metabolism	2p13	1.6

The expression of more than 8,500 well-characterized genes was investigated using the Human Genome Focus Array in the drug-sensitive T-ALL MOLT-4 cell and AraG-resistant variant MOLT-4/AraG900. Data were analyzed using GeneSpring GX11.0 and the robust multichip averaging algorithm. Differentially expressed genes were found using Welch's *t*-test, followed by the Benjamin-Hochberg post hoc test, with a cutoff limit ≤ 0.05. Table shows the eight most significantly up-regulated genes as well as genes involved in nucleoside analog resistance (DCK, DGUOK (dGK), ABCB1), genes known to be involved in fetal hemoglobin regulation (BCL11A and BCL11B, KLF1), and genes involved in hemoglobin degradation or transportation (BLVRB, ABCB6). *FC* fold change

However, the expression of dGK was elevated twofold compared with parental cells, while the activity of dGK measured with AraG as substrate was approximately 10% of the activity in parental cells.

Microarray expression data analysis

The data analysis revealed 1,223 genes with an at least two-fold differential expression between the MOLT-4 and MOLT-4/AraG900 cells. On considering the most up-regulated genes, several fetal hemoglobin genes were represented, as well as the ABCB1 gene (Table 1) encoding for the membrane transporter P-gp involved in multidrug resistance [10]. Up-regulated genes also included KLF1, involved in hemoglobin gamma expression [11], and BLVRB, encoding biliverdin reductase B, which is involved in hemoglobin degradation. The genes BCL11A and BCL11B, probably involved in the regulation of fetal hemoglobin gene expression [12], were 10- and 12-fold down-regulated, respectively (Table 1). The expression of the nucleoside analog–metabolizing enzyme, dCK, was moderately down-regulated, and the expression of dGK was 1.6-fold elevated and was not included in the list of significantly altered genes. The list of significantly altered genes did not reveal any changes in other genes traditionally involved in the resistance mechanisms against AraG or any other nucleoside analogs.

Hemoglobin induction and hypomethylation

In order to determine whether AraG could induce fetal hemoglobin and ABCB1 expression in the short-term perspective and whether that was associated with global DNA hypomethylation, MOLT-4 cells and A549 cells were incubated for eight passages with AraG and 6-MP, the latter being known to hypomethylate DNA [13]. Pulse-labeling of DNA was then performed to assess global methylation, and RNA was prepared to measure hemoglobin gamma expression. Since the MOLT-4 and MOLT-4/AraG900 cell lines had been cultured for a long period of time (up to 6 months during resistance development), new MOLT-4 cells (cultured less than 10 passages since received from ATCC) were used for these experiments. As shown in Table 2, hemoglobin gamma was induced in both MOLT-4 and A549 cells treated with AraG or 6-MP, and so was the ABCB1 gene in both cell lines treated with AraG. The same was seen in the MOLT-4/AraG900 cell line used in the microarray experiment and in a 6-MP-resistant variant generated previously by us (Table 2).

Global methylation was significantly decreased in both cell lines incubated with AraG but only significantly decreased in the A549 cells when incubated with 6-MP (Fig. 1).

Table 2 Hemoglobin gamma and ABCB1 gene expression

Cell line	Treatment (μM)	Hb-gamma FC induction	ABCB1 FC induction
MOLT-4	AraG (5)	30	Induced*
MOLT-4	6-MP (5)	15	n.d.
A549	AraG(lO)	5	74
A549	6-MP (10)	48	n.d.
MOLT-4 parental**	None	n.d.	n.d.
MOLT-4/AraG900**	AraG (0.9)	Induced*	Induced*
MOLT-4/6-MP	6-MP (5)	Induced*	Induced*

MOLT-4 and A549 cell lines were incubated with AraG or 6-MP during 4 weeks, and the expression of hemoglobin gamma and ABCB1 was measured using real-time PCR (mean value from two individual experiments). The expression of these two genes was also evaluated in MOLT-4 parental cells and MOLT-4 AraG900-resistant cells used in microarray experiments, and in MOLT-4 cells resistant to 6-MP. * not detectable in parental cells, **cell lines used in the microarray experiment. *FC* fold change

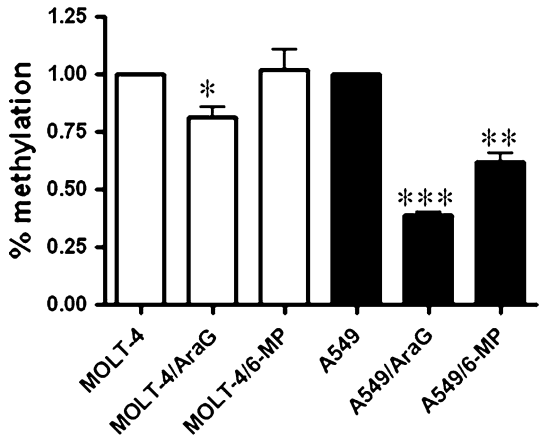

Fig. 1 Global methylation status was investigated in MOLT-4 cells and A549 cells cultured without drug and with AraG or 6-MP. Methylation was assessed using a DNA pulse-labeling technique with thymidine and methionine measuring methylated cytosine in newly synthesized DNA

Western blot analysis of hemoglobin gamma

In the MOLT-4/AraG900 samples used in the gene expression microarray analysis, hemoglobin gamma protein expression was induced as well as in the 6-MP-resistant MOLT-4 cells (Fig. 2). We have previously shown that the P-gp protein expression is induced in these cells as well [4]. These alterations could not be detected in the MOLT-4 and A549 cells that had been incubated with drugs for a short period of time. This is probably due to the shorter induction time and the fact that these proteins are not normally expressed in these cells, and so it may take a higher amount of protein in order for them to be detectable using these antibodies.

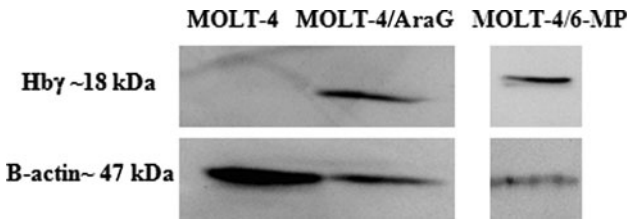

Fig. 2 Protein expression of hemoglobin gamma (Hbγ) was investigated in the MOLT-4 and MOLT-4 AraG and 6-MP-resistant cells using western blot. Hemoglobin gamma protein was highly induced in the resistant cells and compared with β-actin expression

Altered ABCB1 and hemoglobin gamma expression after P-gp inhibition

Several cytotoxic drugs can induce P-gp expression even though they are not substrates of this efflux pump, and many drugs also induce fetal hemoglobin. To investigate whether there is a connection between P-pg and fetal hemoglobin induction, AraG-resistant MOLT-4 cells and K562 cells, the latter known to constitutively express both fetal hemoglobin [14] and low amounts of P-gp [15], were incubated with two inhibitors of this pump. The cells were incubated with cyclosporin A or laniquidar at different concentrations for 96 h in order to see whether the P-gp efflux pump might be involved in the excretion of hemoglobin or its degradation products. Cells were then counted in an automated Beckman Coulter Counter (Beckman Coulter, Inc., Brea, CA, USA), and the expression of hemoglobin gamma and ABCB1 was measured.

All three concentrations tested reduced cell growth of the two cell lines (Fig. 3). RNA was extracted from the cells incubated with the highest concentration of the drugs (10 μM) and analyzed for hemoglobin gamma and ABCB1 expression. We speculated that inhibiting P-gp would lead to toxic concentrations of hemoglobin or waste products of hemoglobin and slow cell proliferation and possibly affect the expression of these genes. On inhibiting P-gp, hemoglobin gamma expression decreased to 5% of control in MOLT/AraG900 cells and to 15–21% in K562 cells (Table 3), while the expression of ABCB1 was markedly increased in the MOLT-4/AraG900 cells, possibly as a compensation for the inhibition.

Discussion

In the present study, using gene expression microarray analysis, we have demonstrated that fetal hemoglobin genes as well as the drug efflux pump, ABCB1, involved in multidrug resistance are up-regulated in the AraG-resistant variant of the human leukemic MOLT-4 cell line. We expected

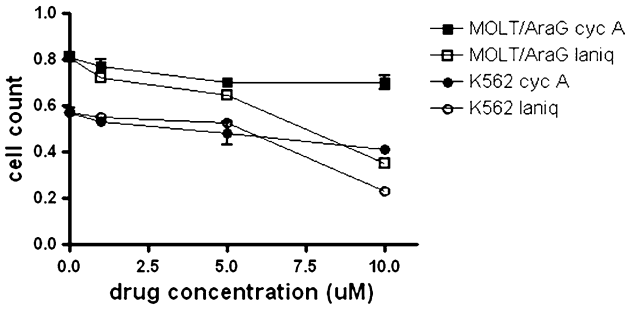

Fig. 3 The resistant MOLT-4/AraG900 and K562 cells were incubated during 96 h with the P-gp inhibitors cyclosporin A (cyc A) and laniquidar (laniq) at different concentrations (1, 5, 10 μM). Cells were then counted, and a graph was plotted showing growth inhibition of both drugs at all concentrations. Y-axis showing amount of million cells/ml

Table 3 Hbγ and ABCB1 expression after P-gp inhibition

Treatment	MOLT-4/AraG900 (% of control)		K562 (% of control)	
	Hbγ	ABCB1	Hbγ	ABCB1
cyc A 10 μM	5	>300	21	93
laniq 10 μM	5	>300	15	140

MOLT-4/AraG900 and K562 cells, both expressing fetal hemoglobin and the drug efflux pump P-gp, encoded by ABCB1 were incubated for 96 h with two P-gp inhibitors, cyclosporin A (cyc A), and laniquidar (laniq). mRNA expression of hemoglobin gamma (Hby) and ABCB1 was then assessed using real-time PCR and presented as the percent of controls (cells cultured with no drug). Data show the mean from two experiments

major changes in genes involved in nucleoside analog resistance, such as nucleoside analog–metabolizing enzymes, and hoped to obtain information about additional genes involved in the mechanisms of resistance. However, genes known to be involved in resistance development were only moderately affected together with changes in more than 1,000 additional genes. The reason for these unexpected results is not entirely clear. It may be due to the fact that the cells were selected for resistance during several months and that the mechanisms for resistance change over time. It may also be because the concentrations used for resistance induction are of such a nature that low concentrations induce certain resistance mechanisms and higher concentrations induce other mechanisms. Also, the resistance mechanisms may not be reflected by the mRNA expression of the genes. The fact that the mRNA expression of dGK, which is known to phosphorylate and activate AraG, was, in fact, slightly induced, while the enzymatic activity was one-tenth of that in control cells, suggests post-translational changes not seen at the mRNA level.

Fetal hemoglobin [16] and ABCB1 gene induction [17, 18] is correlated with hypomethylation of DNA, and we speculated that AraG has hypomethylating properties since these genes were induced in AraG-resistant cells. This was investigated in MOLT-4 cells and the lung adenocarcinoma cell line A549 by means of a four-week drug induction with AraG and the antimetabolite 6-MP, a drug known to hypomethylate DNA [13] and, like AraG, is incorporated into DNA. We used the A549 as a control cell line since many nucleoside analogs, and particularly AraG, have a very modest effect on solid tumors, and we wanted to see whether the effects of AraG were applicable to other cells than leukemic. Fetal hemoglobin gamma was induced in both cell lines, while ABCB1 was induced in cells treated with AraG only. The global methylation of DNA was also significantly decreased in both cell lines treated with AraG. Drug concentrations that inhibited approximately 50% cell growth in the cultured cells were used since we wanted to mimic the development of resistance when some cells more tolerant to the treatment survive and expand into a less drug-sensitive population. The effect on DNA methylation may depend on drug concentrations, exposure times, and cell lines used.

The gamma-globin gene is silenced through methylation shortly after birth, a process known as the fetal switch, but can easily be re-expressed after treatment with demethylating agents [19, 20]. Substances shown to induce fetal hemoglobin in cell lines include sodium butyrate, cisplatin, imatinib, cytosine arabinoside, and hydroxyurea [14, 21–27], 5-azacytidine, and decitabine [28–31]. Two genes, BCL11A and BCL11B, working as transcriptional repressors of the fetal hemoglobin genes [12, 32] were down-regulated in the resistant cells. Therefore, transcriptional repression of these two genes may also be one explanation for the massive fetal hemoglobin induction.

The expression of the nucleoside analog–activating enzymes dCK and dGK was only moderately altered according to the microarray analysis and real-time PCR measurements, while enzymatic activities were reduced. However, we have previously demonstrated a lack of correlation between mRNA expression and the enzymatic activity of these enzymes [33]. The multidrug resistance gene, ABCB1, was one of the most up-regulated genes. We have shown before that this gene is induced in MOLT-4 cells selected for resistance to AraG [4] and is probably responsible for the cross-resistance to daunorubicin seen in these cells. We have also studied the K562 cell line and a variant resistant to vincristine, a substrate of P-gp [34]. The presence of ABCB1 and P-gp expression after drug withdrawal was evaluated over 11 months. These levels remained unchanged for 5 months without vincristine, but then rapidly decreased, at which time cells also became more sensitive to P-gp substrates [34]. However, these cells remained highly resistant to AraG. It was shown that after 11 months of drug withdrawal, some cells still contained low levels of ABCB1/P-gp which were easily re-induced, [34] and we

showed that AraG had the ability to re-induce P-gp during short-term incubations. Like other nucleoside analogs, AraG is not considered to be a substrate of P-gp [35] but can yet induce P-gp expression. Prenkert et al. showed that 10-min incubation with 0.5 μM of the nucleoside analog cytarabine induced a 1.7-fold increase in ABCB1 expression in human HL60 cells, while, for daunorubicin, which is a P-gp substrate [35], a 1.6-fold increased ABCB1 induction was not seen until after 24 h [36]. It has previously been shown that HL60 cells resistant to the anthracycline doxorubicin had increased P-pg expression and showed cross-resistance to AraC, and upon inhibition of P-gp with cyclosporine A, the intracellular retention of AraC was increased [37]. The authors therefore speculated that resistance to AraC in these cells was due to both decreased AraC activation by dCK which was slightly reduced and efflux of AraC by P-gp. This may be the reason for AraG and nucleoside analog resistance in general, in the present study as well. Studies have also shown that AraC can activate the protein kinase C (PKC) signaling pathway [38, 39] and the mitogen-activated protein (MAP) kinase p38, [39] eventually leading to activation of AP-1 which is the transcription factor for the ABCB1 gene and the hemoglobin gene locus [40, 41]. Activation of p38 kinase is also activated by the hypomethylating cytosine analog decitabine, [42] and this intracellular pathway seems to be a common mechanism for hypomethylating drugs and possibly shared by AraG as well.

It is tempting to speculate that ABCB1 expression is induced in response to AraG and other hypomethylating drugs due to the increased hemoglobin concentration in the cells since when hemoglobin is degraded and excreted, different P-gp transporters are expected to be involved in its transportation [43] and also since inhibitors of P-gp increase bilirubin entry into the rat brain [44]. In that case, this would explain why nucleoside analog–resistant and other resistant cells show a multidrug-resistant phenotype overexpressing P-gp. On analyzing the gene expression microarray analysis data, we also found up-regulated genes involved in hemoglobin synthesis and degradation. The ABCB6 gene encodes a mitochondrial transporter required for mitochondrial porphyrin uptake necessary for hemoglobin synthesis [45], and this gene was slightly up-regulated, as well as ABCG2 (not shown) also involved in this transport [46]. On inhibiting P-gp in the MOLT-4/AraG900 and the K562 cells, the expression of hemoglobin gamma was decreased, and in the MOLT-4/AraG900 cells, the expression of ABCB1 was greatly induced. K562 cells constitutively express low levels of P-gp [15, 47] and fetal hemoglobin, [15] and this is probably the reason for the modest effect on ABCB1 and hemoglobin gamma expressions after P-gp inhibition in this cell line. We speculate that this may occur due to increased retention of hemoglobin and its degradation products in the cells when inhibiting P-gp, with decreased hemoglobin production and increased P-gp expression in order to try to compensate for the decreased efflux of hemoglobin or hemoglobin waste products. The P-gp inhibitors slightly affected the cell growth in the parental MOLT-4 cells at the highest concentrations used, but since these cells do not express P-gp this effect must be due to some other actions of the drugs or possibly off-target effects due to the high concentration used.

One study showed that short-term treatment (96 h) with different cytotoxic drugs induced ABCB1 mRNA expression in K562 cells, but that this was due to mRNA stabilization which did not lead to P-gp expression at the membrane surface, but that long-term selection for drug resistance did lead to mRNA association with polyribosomes and translation of P-pg [47]. We have previously characterized the MOLT-4 and the MOLT-4/AraG900 cells with regard to P-gp expression. These experiments demonstrated an absence of P-gp in MOLT-4 cells, but a high expression in the AraG-resistant cells measured by western blot, [4] and we therefore assume that the massive up-regulation of ABCB1 seen in this study is accompanied by an increased P-pg expression. However, the fact that AraG induced ABCB1 expression in both MOLT-4 and A549 cells during short-term treatment, along with hemoglobin gamma expression, suggests that AraG and other DNA-hypomethylating drugs should be used with caution together with P-gp substrate drugs due to the ABCB1 induction.

Leukemia arises partly due to inactivation of genes controlling differentiation and growth [48], and this may be a consequence of epigenetic silencing of the genes. These genes may become reactivated by such inhibitors of DNA methylation as 5-aza-2′-deoxycytidine or, possibly, AraG. It would be interesting to find out what genes are hypomethylated and re-expressed in patients after short-term AraG treatment and whether these genes could be used for targeted therapies, or if they only render the patients less susceptible to therapy by inducing genes involved in nucleoside analog resistance.

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