Optimization and evaluation of electroporation delivery of siRNA in the human leukemic CEM cell line

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Abstract In order to study nucleoside analog activation in the CEM cell line, a transfection protocol had to be optimized in order to silence an enzyme involved in nucleoside analog activation. Hematopoietic cell lines can be difficult to transfect with traditional lipid-based transfection, so the electroporation technique was used. Field strength, pulse length, temperature, electroporation media, siRNA concentration, among other conditions were tested in order to obtain approximately 70–80% mRNA and enzyme activity downregulation of the cytosolic enzyme deoxycytidine kinase (dCK), necessary for nucleoside analog activation. Downregulation was assessed at mRNA and enzyme activity levels. After optimizing the protocol, a microarray analysis was performed in order to investigate whether the downregulation was specific. Additionally two genes were differentially expressed besides the downregulation of dCK. These were however of unknown function. The leakage of intracellular nucleotides was also addressed in the electroporated cells since it can affect the DNA repair mechanism and the efficiency

of nucleoside analogs. Three of these pools were increased compared to untreated, unelectroporated cells. The siRNA transfected cells with reduced dCK expression and activity showed reduced sensitivity to several nucleoside analogs as expected. The multi-drug resistance to other drugs, as seen in nucleoside analog-induced resistant cells, was not seen with this model.

Keywords RNAi · Electroporation · Microarray · Deoxycytidine kinase · Nucleoside analog

Introduction

Nucleoside analogs are drugs used for cancer chemotherapy and are prodrugs needed to be phosphorylated intracellular in order to become active. The cytosolic enzyme deoxycytidine kinase (dCK, E.C.2.7.1.74) activates both natural nucleosides and nucleoside analogs to monophosphates that can further be phosphorylated to di- and triphosphates (Arner and Eriksson 1995). Decreasing the expression of this enzyme experimentally may help in the understanding of resistance mechanisms to nucleoside analogs, since dCK activity and expression often are reduced in nucleoside analog-resistant patients and cell lines (Bai et al. 1998; Lotfi et al. 1999). Nucleoside analogs exert their effect by incorporating into growing DNA, terminating the synthesis, and by disturbing RNA

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synthesis or the enzyme ribonucleotide reductase. This enzyme is involved in ribo- and deoxyribonucleotide pool maintenance by reducing ribonucleotides (NTPs) to the corresponding deoxyribonucleotides (dNTPs). The intracellular nucleotides and the nucleotides of the activated nucleoside analog compete for the same incorporation sites in the cell. The ribonucleotide reductase is made up of two non-identical subunits called R1 and R2. The R1 protein is expressed constantly and predominantly in proliferating cells, while the smaller R2 protein is cell cycle regulated (Nordlund and Reichard 2006). Another protein called p53-induced R2 protein (p53R2) has been shown to form an active RR complex with the R1 subunit in G1- or non-proliferating cells (Guittet et al. 2001; Wang et al. 2009). Nucleoside analog resistance can sometimes be explained by increased expression of one or several of these subunits leading to increased endogenous dNTPs competing with the active nucleoside analogs for the same place of incorporation (Bergman et al. 2005; Goan et al. 1999; Mansson et al. 2003).

To study the intracellular activation of nucleoside analogs to deoxynucleotides, we decided to down-regulate the dCK enzyme using siRNA. Electroporation was chosen to transiently transfect and downregulate dCK in the human T-cell acute lymphoblastic leukemia CEM cell line. This cell line was chosen because it, like other hematopoietic cells, expresses high levels of dCK, and since nucleoside analogs primarily are used to treat different types of leukemias (Fox et al. 1991). There were limited data available on transient siRNA transfection in CEM cells using electroporation, and therefore attempts were carried out to optimize a protocol addressing this. The gene expressions in untreated CEM cells, electroporated cells, and siRNA-treated cells were also evaluated using whole-genome microarray analysis. Cytotoxicity measurements, using several clinically relevant nucleoside analogs, were performed comparing dCK siRNA- and negative control transfected cells.

Materials and methods

Materials

Cells were purchased from American Type Culture Collection (Rockville, MD, US), while cladribine (Leustatin[®]) was from Johnson & Johnson

(Schaffhausen, Germany), cytarabine (Cytosar[®]) was from Pharmacia & Upjohn (Stockholm, Sweden), clofarabine (2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, Evoltra[®]), was a gift from Dr. Howard Cottam, University of California (San Diego, CA, USA), nelarabine (9- β -D-arabinofuranosylguanine, Atriance[®]) was from R.I. Chemical (Orange, CA, USA), fludarabine (2-fluoroarabinosyladenine, Fludara[®]) was kindly provided by Dr. Zeév Shaked, Berlex (Alameda, CA, USA), and daunorubicin (Cerubidin[®]) was from Rhône-Poulenc Rorer (Bristol, UK). Electroporation devices and chemicals were from Bio-Rad Laboratories (Hercules, CA, USA), cell culturing reagents were from Gibco, Life Technologies (Paisley, UK), siRNAs were from Ambion, Inc. (Huntington, Cambridge, UK), real-time PCR equipment and reagents were from Applied Biosystems (Foster City, CA, USA), microarray reagents and equipment were from Affymetrix (Buckinghamshire, UK), and additional chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden).

Cells and siRNA transfection

CEM cells (acute T-lymphoblastic leukemia) were kept in RPMI-1640 medium with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μ g/ml streptomycin (PEST), and 2 mM L-glutamine. Prior to siRNA transfection cells were washed in RPMI-1640 medium and resuspended in the same medium. Cells were electroporated in 0.4 cm cuvettes with a Gene Pulser Xcell Electroporation System. Small interfering RNAs used were the *Silencer*[®] Validated siRNA # 68, #69 and #70 for human deoxycytidine kinase. *Silencer*[®] negative control siRNA that did not bind to any human mRNA sequences was used and transfected into control cells, and *Silencer*[®] GAPDH siRNA was used as a positive control during optimization of the transfection. Optimization parameters are presented in Table 1, and final transfection protocol is presented in the “Results” section. The CEM cells used were an average of 13 μ m in diameter and the appropriate range of voltage to be tested was estimated using this formula (www.eppendorf.com):

$$E_C = V_C / (0.75 \times d_{\text{cell}})$$

where

E_C critical field strength (V/cm)

Table 1 Optimization of electroporation parameters

Voltage–time	Expression		Activity	Viability	
	ED	SW		ED	SW
300 V–10 ms ^a	87	80		~ 60	~ 60
340 V–10 ms	75	60		~ 60	~ 60
360 V–10 ms	80	74		~ 60	~ 60
400 V–10 ms	75	70		~ 60	~ 60
440 V–10 ms	80	85		~ 50	~ 50
SW, 340 V, siRNA #68 250 nM ^a					
	1 Pulse	2 Pulses			
5 ms 1/2 pulse(s)	65	60			~ 60
10 ms 1/2 pulse(s)	60	50			~ 60
15 ms 1/2 pulse(s)	60	60			~ 60
SW, 340 V, 10 ms 2, siRNA #68 250 nM ^a					
4 Degrees		52			~ 60
Room temperature ^b		50			~ 60
0.4 ml ^b		48			~ 60
0.8 ml		54			~ 60
1 ml/ml		50			~ 60
4 ml/ml ^b		45			~ 60
8 ml/ml		67			~ 60
340 V, 10 ms × 2					
125 nM (#68-#69-#70)		82-67-77			~ 60
250 nM (#68, #69, #70)		Fig. 1a	Fig. 1a		~ 60
500 nM (#68-#69- #70)		50-42-23			~ 60
340 V, 10 ms × 2, 250 nM #70					
PBS	27		42		~ 60
RPMI-1640 ^b	24		38		~ 60
PEST	39		49		~ 60
–PEST	28		41		~ 60
+FCS p.e +DMSO	27		25		~ 90
–FCS p.e –DMSO	32		40		~ 65

Different parameters were tested in order to optimize siRNA delivery into CEM cells. Transfection efficiency was evaluated by measuring dCK mRNA levels 24 h after transfection as well as cell viability. dCK enzyme activity was measured when mRNA levels were reduced with approximately 50%. Mean from at least two experiments

p.e post-electroporation, ED exponential decay, SW square waves. Blank lanes indicate data not provided

^a Recommendations from the literature

^b Conditions used during optimization if not otherwise indicated

V_C permeation voltage of the membrane (1 V at 20 °C, 2 V at 4 °C)

d_{cell} cell diameter (cm)

and $V = E_C \times d_{\text{cuv}}$

where

V voltage setting on electroporator

d_{cuv} electrode distance (gap width, cm)

RNA extraction and PCR

RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Solna, Sweden). Complementary DNA was produced using the High Capacity Archive Kit (Applied Biosystems) according to the manufacturer's instructions. Primers and probe for dCK and the housekeeping gene β_2 -microglobulin, as well as Taqman[®] Gene Expression Assays (20X) for deoxyguanosine kinase (dGK), thymidine kinase 2 (TK2), human equilibrative nucleoside transporter 1 (hENT1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH,) are shown in Table 2. Reactions were carried out in a 7,500 Fast Real-time PCR System.

Protein extraction and enzyme activity measurements

Cells were centrifuged at 600g and washed three times with ice-cold PBS, and cell pellets were resuspended in a protein extraction buffer (50 mM Tris-HCl (pH 7.6), 4 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.5% Nonidet P40) and lysed in liquid nitrogen. After a 15 min centrifugation at 15,000g, supernatants were

collected and assayed for dCK activity as previously described by (Fyrberg et al. 2007) using 10 μ M deoxycytidine ([8-³H]-2'-deoxycytidine, specific activity 8.3 Ci/mmol) as substrate. Activities were expressed as pmol of formed product per min per mg of protein and protein contents (mg/ml) were determined by the method of Lowry et al. (1951) using the DC protein assay (Bio-Rad Laboratories).

Drug cytotoxicity assay

The methyl thiazole tetrazolium (MTT) assay (Mosmann 1983) was used to assess nucleoside analog cytotoxicity in siRNA transfected cells as described in (Fyrberg et al. 2007). After siRNA transfection, the cells were allowed to recover for 24 h before initiating the experiments, and drug incubations were carried out during 48 h. Absorbance was measured at a wavelength of 540 nm with the reference at 650 nm and an IC_{50} value was calculated as the percent survival compared to control cells.

Western blot

Protein expressions were visualized using Western blot. Forty μ g of proteins were loaded on a Criterion Precast 4–15% Tris-HCl gel and run in a sodium dodecyl sulphate (SDS) running buffer (0.4 M glycine, 50 mM Tris-HCl, pH 8.5, 14 mM SDS) at 100 V for 2 h. The Precision Plus Protein Kaleidoscope[™] Standard was used to determine molecular weight. Proteins were transferred onto an Immune Blot PVDF membrane in a transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) at 50 V for 1 h. A monoclonal mouse anti-R1 antibody (AD 03, 1:1000) was from InRo Biomedtek (Umeå, Sweden),

Table 2 Primers, probes and TaqMan 20 × assays

Primers, probes, and 20× assays used to measure gene expression using real-time PCR	dCK	F:5'-CCACCCCGCCCAAGA-3', R: 5'-CCTCCCTGCAGCGATGTTC-3', P:5'-6-FAM-CTTTC^CAGCCAGCTCTGAGGGGACCTT-3'
	β_2 -microglobulin	F:5'-AGGACTGGTCTTCTATCTCTTGACTACAC-3', R:5'-AAGTCACATGGTTCACACGCG-3', P:5'-VIC-TCACCCCACTGAAAAAGATGAGTATGCCT-3'-TAMRA
	dGK	Hs00365632_m1
	TK2	Hs00936915_m1
	hENT1	Hs01085709_g1
^ denotes dark quencher in the dCK probe	GAPDH	Hs00266705_g1

polyclonal rat anti-R2 antibody (1:1000) was kindly provided by Dr. D. Lembo at the Department of Public Health and Microbiology, University of Turin (Lembo et al. 2000), polyclonal rabbit anti-p53R2 (ab8105, 1:1000) and polyclonal rabbit anti- β -actin (ab8227, 1:1000) were from Abcam (Cambridge, UK). 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, US).

HPLC analysis of intracellular nucleotide pools

Five million CEM cells were harvested at 600g and washed three times in ice-cold PBS. The cell pellets were resuspended in 200 μ l H₂O and boiled for 5 min. After a centrifugation at 15,000g for 15 min, the supernatants were collected and analyzed for NTP pool content on a Purospher Star 4 $\times$ 55 mm column (Waters) at 30 °C. Twenty-five μ l of the sample was injected onto the column and eluted at a flow rate of 1.23 ml/min using a 0–25% gradient with acetonitrile and 20 mM K₂HPO₄ buffer (20 mM K₂HPO₄, pH 7.0, 2.1 mM tetrabutylammonium bisulphate, 3% acetonitrile). A mixture of all ribonucleotides with known concentrations was used as a reference. The amount of each NTP pool was calculated by comparing the area under the curve/height with a standard. The concentrations of GTP and ATP were measured at 262 nm and UTP and CTP were monitored at 271 nm.

Microarray analysis

The GeneChip[®] Human Gene 1.0 ST Array was used and RNA from one representative experiment was analyzed using these arrays. Samples were prepared according to the manufacturer's instructions. Chip data were analyzed using the GeneSpring GX[®] 11.0 Software (Agilent Technologies, Sweden AB). Background correction, normalization and probe summarization were done using the robust multichip averaging (RMA) algorithm (Irizarry et al. 2003), and significantly differentially expressed genes were found using Welch's *t*-test followed by Benjamin-Hochberg post hoc test. Samples were filtered on expression, *p*-value computation was asymptotic, and the cut-off limit was drawn at 0.05. DCK and three additional genes from the DNA chip were verified using real-time PCR.

Results

Optimizing electroporation parameters

Different parameters were tested in order to optimize the electroporation conditions (Table 1). Cell viability was evaluated by counting the cells, 1 h after transfection at the earliest in an automated Beckman Coulter Counter (Beckman Coulter, Inc., Brea, CA, USA). Transfection efficiency was evaluated measuring dCK mRNA expression at first, and later on also the dCK enzyme activity. Square waves were superior to exponential decay for transfection efficiency but not for survival, and increased the amount of transfected cells with approximately 15% from an initially 25% dCK mRNA down-regulation to 40% dCK mRNA down-regulation compared to control cells. Different voltage, pulse length and number of pulses were also evaluated, as well as temperature, cell density and cuvette volume. Optimizing these parameters slightly enhanced the transfection efficiency but had no effect on cell viability (Table 1).

Three different validated siRNAs targeting the dCK mRNA were evaluated, and the siRNA #70 was the optimal in these cells and led to approximately 40% dCK down-regulation compared to control cells of the activity but more pronounced down-regulation of the mRNA expression (Fig. 1a). This siRNA had equal or slightly higher effect when used at 500 nM but to avoid off-target effects of the siRNA, the lower concentration of 250 nM was chosen. Adding 1.25% DMSO to the cells before electroporation, and 20% FCS just after electroporation increased the transfection efficiency with approximately 15% to 75% dCK down-regulation at the mRNA and enzyme activity level compared to control cells. DMSO and FCS had a pronounced effect on cell viability, and reduced cell death to approximately 90% viable cells compared to 60%. DMSO may affect the cells negatively, and therefore dCK expression was monitored in cells electroporated with or without 1.25% DMSO (without siRNA) and compared to the dCK expression in untreated logarithmically grown CEM cells. As depicted in Fig. 1b, there were basically no differences in dCK expression between the two conditions. Another siRNA was also tested, and this was a validated positive control against GAPDH. The downregulation of GAPDH was also highly efficient in the CEM cells (Fig. 1c). Cell viability was estimated by counting the cells

Fig. 1 **a** Three different validated siRNAs (250 nM) against dCK were evaluated by measuring mRNA expression and enzyme activity in CEM cells 24 h after electroporation. **b** The effect of 1.25% DMSO on the expression of dCK was measured in cells electroporated with or without DMSO and compared to the dCK expression in logarithmically grown untreated CEM cells (% of control meaning compared to logarithmically grown, untreated CEM cells, mean of two

experiments). **c** The effect of dCK downregulation was measured at the mRNA level after using dCK (filled square bracket) or positive control GAPDH (open square bracket) siRNA in CEM cells. Mean of two different experiments. **d** Three or more experiments were performed to evaluate the optimized electroporation method using dCK siRNA in CEM cells

approximately 1 h after electroporation and then after 24, 48, and 72 h. Cell number was only moderately affected using the final optimized protocol but cells proliferated more slowly up to 48 h after the electroporation procedure compared to untreated CEM cells.

Several experiments were conducted using the final optimized protocol. Cells (4×10^6 cells/ml) were electroporated in RPMI-1640 supplemented with 1.25% DMSO and 125 nM siRNA (#70) at room temperature in 0.4 cm cuvettes in a volume of 400 μ l. They were pulsed at 340 V for 10 ms, repeated once after 10 s. Cells were rinsed out of the cuvette using a pre-heated (37 °C) post-electroporation medium containing RPMI-1640, L-glutamine (2 mM) and 20% FCS. Using this protocol, the dCK downregulation was approximately 75% of control at mRNA and activity level at 24 h, then rose, but was present up to 72 h (Fig. 1d).

Cytotoxicity measurement in siRNA transfected cells

Cytotoxicity measurements were performed in siRNA transfected cells using MTT assay to evaluate the effect of dCK downregulation. The sensitivity to the nucleoside analogs cladribine, cytarabine, and clofarabine were three- to six-fold decreased in cells transfected with dCK siRNA compared to cells transfected with negative control siRNA (Table 3) with clofarabine being most dependent on dCK phosphorylation and cladribine the least dependent of these three. The nucleoside analogs fludarabine and nelarabine showed unaltered toxicity in the cells after dCK down-regulation (Table 3). The cytotoxicity to the anthracycline daunorubicin was unchanged since its activity is independent of dCK.

Table 3 Cytotoxicity data in siRNA transfected cells

	Resistance factor
Danorubicine	1.5 ± 0.7
Nelarabine	0.9 ± 0.6
Fludarabine	0.9 ± 0.6
Cladribine	3.3 ± 1.8
Cytarabine	4.8 ± 1.8
Clofarabine	6.0 ± 3.0

Cytotoxicity to different drugs was evaluated after dCK downregulation using the MTT assay. Resistance factor: IC₅₀-value in cells transfected with dCK siRNA/IC₅₀-value of cells transfected with negative control siRNA

Expression of ribonucleotide reductase subunits

Levels of the ribonucleotide reductase subunits were measured using western blot. This was done since altered levels of this enzyme may also confer resistance against nucleoside analogs, and we wanted to make sure that altered sensitivity to nucleoside analogs was not due to changes in this enzyme. The subunits, R1, R2, and p53R2 were not significantly altered in CEM cells treated with dCK siRNA as compared to negative control transfected cells, and in relation to β -actin expression (Fig. 2).

Measuring nucleotide leakage from electroporated cells

If cells are substantially damaged during the electroporation process they may leak intracellular nucleotides that are required by the cells for repairing DNA

Fig. 2 The three subunits of the ribonucleotide reductase enzyme was measured using western blot but their level was not altered in the dCK siRNA treated cells compared to negative control transfected cells and the loading control β -actin

breaks that can be induced by the electric pulse, and it may also make the cells more sensitive to nucleoside analogs since the NTP and dNTP pools are closely regulated by the ribonucleotide reductase enzyme (Plunkett and Grindey 1987; Xie and Plunkett 1996). However, pulses above 900 V/cm have not been able to induce leakage of nucleotides sufficient to interfere with DNA repair synthesis (Meldrum et al. 1994). The intracellular nucleotide pools in electroporated CEM cells were compared to untreated CEM cells 24 h after electroporation. GTP pools were reduced up to two-fold in electroporated cells compared to untreated CEM cells, while CTP, UTP and ATP pools were elevated three-, ten- and 1.3-fold, respectively (Fig. 3a). All NTP pools were reduced in dCK siRNA transfected cells compared to negative control transfected cells at 24 h after electroporation (Fig. 3b) but had normalized again after 96 h (Fig. 3c).

Whole-genome microarray analysis of electroporated cells

A genome-wide microarray was performed 48 h post-electroporation. This was done in order to see the effects of the electroporation procedure on the cells, and the effects of the negative control siRNA and the dCK siRNA on the cells. We wanted to see if the dCK down-regulation was specific or if the siRNA had off-target effects on any other genes. The time-point of 48 h was chosen because it was the time-point in the middle of the cytotoxicity measurement with nucleoside analogs. We also wanted genes affected by the electroporation procedure itself to stabilize and to see effects on genes exerted by the siRNA only. When comparing untreated CEM cells compared to electroporated CEM cells, a total of 124 genes were differentially expressed when looking above a two-fold difference in gene expression. When changing the settings to above four-fold difference a total of 10 genes were upregulated. There were genes involved in apoptosis, fatty acid metabolism, cholesterol biosynthesis, and glucose metabolism (Table 4). When comparing the electroporated cells with cells transfected with the negative control siRNA, one gene was differentially expressed corresponding to the complete genome of the mitochondrion (Table 4). When comparing dCK siRNA transfected cells to the negative control cells the cut-off was drawn at 1.7-fold difference since that was

Fig. 3 **a** Leak of nucleotides was examined in electroporated cells using HPLC, and nucleotide pool content was compared to untreated logarithmically grown CEM cells. At 24 h after electroporation no leakage occurred, instead the UTP pool was markedly increased. X-fold difference on Y-axis **(b)** Nucleotide pool content at 24 h after electroporation in cells transfected with dCK siRNA was compared to cells transfected with negative control siRNA. All pools were slightly reduced but had normalized again or were slightly increased 96 h after transfection **(c)**.

the level of dCK downregulation, and this was also the only gene down-regulated. At this cut-off, additionally two genes were upregulated. However, these were genes with unknown function or with very little knowledge about (Table 4), and hence it is not

possible to speculate about their potential role in dCK downregulation.

In addition to dCK, three other genes were examined and verified using realtime-PCR in relation to the microarray expression. The genes of two additional kinases also able to activate some nucleosides and nucleoside analogs, dGK and TK2, were examined. The expression of a nucleoside transporter involved in transportation of nucleoside analogs across the cell membrane, called hENT1, was also examined in order to see if altered expression of these genes could help explain part of the reduced activation of nucleoside analogs. DCK expression was 43% of control using realtime-PCR, while for dGK, TK2, and hENT1 it was 118, 130, and 104% of control, respectively. These data were more or less in agreement with the chip data and these enzymes/transporters were probably not involved in any nucleoside analog resistance since we then had expected altered levels of these.

Discussion

Electroporation has direct toxic effect on cells, but so has the lipid-mediated transfection reagents as well, and by optimizing the parameters used during transfection one can avoid toxicity and still receive high transfection efficiency. Two types of pulses can be applied, the exponential decay which decreases during time and square waves, which allows a controlled pulse of fixed amplitude for a fixed period of time. In these experiments transfection efficiency, but not cell viability, was enhanced, using square waves, as has been shown in other studies as well (Liu and Bergan 2001). One would also like to apply sufficiently high enough voltage to permeabilize cells without a major cell loss. The threshold for reaching permeabilization is lower the larger the cell, and since cells grown in culture do not have the exact same size due to different age, membrane thickness and volume it is virtually impossible to permeate all cells simultaneously (Ferret et al. 2000). The CEM cells used in this study were approximately 13 μm in diameter and most suppliers recommend the voltage settings to be in the range of 200–500 V. The length of the applied pulse is important for permeation, but also has a significant effect on the DNA strand breaks

Table 4 Whole-genome microarray data on electroporated cells

Genbank	fc	Regulation	Genesymbol	Genedescription
CEM vs. electroporated four-fold				
AF097514	5.3	Up	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)
AF002697	5.5	Up	BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3
AF108658	4.8	Up	FADS2	Fatty acid desaturase 2
AB209607	7.8	Up	SLC2A3	Solute carrier family 2 (facilitated glucose transporter)
BC003613	15.5	Up	ALDOC	Aldolase C. fructose-bisphosphate
AF426269	4.3	Up	C21orf90	Chromosome 21 open reading frame 90
AK125918	4.9	Up	GBE1	Glycogen branching enzyme
BC107879	4.6	Up	SC4MOL	Sterol-C4-methyl oxidase-like
BC083514	6.0	Up	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
BC001880	7.0	Up	INSIG1	Insulin induced gene 1
Electroporated vs. neg. control two-fold				
NC_001807	2.1	Down		Mitochondrion complete genome
Negative control vs. dCK 1.7-fold				
BC103764	1.7	Down	DCK	Deoxycytidine kinase
AF495523	1.8	Up	REXO1L1	REX1. RNA exonuclease 1 homolog
BC130374	1.7	Up	GAGE13	G antigen 13

A whole-genome microarray was performed on RNA from untreated CEM cells, electroporated cells, and cells transfected with negative control siRNA or dCK siRNA transfected cells (dCK) 48 h after electroporation/transfection. The table shows the most differentially expressed genes at different thresholds. Fc-fold-change

and cell rupture induced by the electroporation (Meaking et al. 1995).

Adding different reagents to the electroporation media may improve both survival and transfection efficiency substantially. Bahnson and Boggs showed that some low-molecular weight molecule(s) in serum had a sealing effect on electroporated cells and also enhanced the transfection efficiency (Bahnson and Boggs 1990). They showed that 20 min after electroporation at a serum concentration of 20%, virtually all cells were impermeable to lucifer yellow (Bahnson and Boggs 1990). Electroporation with FCS slightly reduced the transfection efficiency, probably due to protein–siRNA binding, and therefore 20% serum was used in the post-electroporation medium since this was shown to increase the downregulation and enhance cell viability. The effect of DMSO on electroporated cells is unclear so the mRNA expression of dCK was measured in cells electroporated with or without DMSO and compared to untreated CEM cells. The expression profiles resembled each other, and the initial drop-down was probably due to

the decreased proliferative capacity of the electroporated cells, since we have shown before that at least the activity of dCK closely follow the S-phase distribution of the cells (Fyrberg et al. 2007). It is speculated that DMSO takes part in the permeation process of membranes and stabilizes the cell membrane so that transfected cells survive after electroporation (Melkonyan et al. 1996), and is also a scavenger of reactive oxygen species (Yu and Quinn 1994) that could potentially harm the fragile electroporated cells. Antibiotics were avoided during electroporation since the intracellular concentration of antibiotics may reach high and potentially toxic levels if allowed to flow into the open pores of electroporated cells.

Three different validated siRNA sequences were tested and data clearly showed that one siRNA (#70) was superior to the other two. A mixture of all three siRNAs did not enhance downregulation further (data not shown). Of randomly chosen siRNAs, 70–90% are able to create an 80% knock-down of the target gene, but since suspension cells are more difficult to

transfect they require even more efficient siRNA. Also, genes with highly abundant protein products or very stable proteins may be difficult to silence with traditional siRNAs (Verma and Dey 2004). A 40–75% downregulation of dCK mRNA and activity, as in this case, was apparently sufficient to see an effect on nucleoside analog activation. dCK expression and activity started to normalize shortly after down-regulation. At 96 h after electroporation, mRNA expression was approximately 75% of control cells, while dCK enzyme activity was 125% of control. This may be an overcompensation mechanism occurring after the repression of the enzyme. Because the cells are highly dependent on dCK for normal function and salvage of nucleosides for DNA synthesis (Arner and Eriksson 1995) they may increase dCK activity in order to make up for its decreased activity during the siRNA suppression.

Three of the nucleoside analogs tested were less toxic to the cells after downregulation of dCK, indicating that they are highly dependent on dCK phosphorylation to become activated. It was not very surprising that nelarabine and fludarabine showed unaltered sensitivity in the cells since nelarabine is also efficiently activated by the mitochondrial enzyme deoxyguanosine kinase (Zhu et al. 2000) and that resistance mechanism against fludarabine has been shown to include altered activity of the ribonucleotide reductase enzyme rather than decreased dCK activity (Mansson et al. 2003). During these experimental conditions both deoxyguanosine kinase and the ribonucleotide enzyme seemed unaffected by the dCK downregulation, which probably is one of the reasons for the sustained cytotoxicity of nelarabine and fludarabine.

Intracellular nucleotide pools were monitored in electroporated cells in order to see if nucleotide leakage occurred, that could possibly affect DNA repair synthesis, and nucleoside analog efficiency. The GTP pool was reduced in the electroporated cells, while the CTP, UTP and ATP pools were increased in the cells. These measurements reflect the NTP content at 24 h post-electroporation and the elevated pools may be seen as an over-compensation of the nucleotide leakage that possibly took place immediately after electroporation. Therefore it may be possible that the intracellular NTP pools would look different choosing another time-point for measuring, since we had expected some degree of NTP

leakage from the electroporated cells. On the other hand we expected the cells to be slightly more vulnerable directly after electroporation and we therefore waited 24 h before preparing them for measuring of NTP content. The reduction of all NTP pools in dCK siRNA transfected cells may be a consequence of the dCK down-regulation. dCK can provide the cell with all four dNTPs needed for DNA synthesis (Arner and Eriksson 1995), and if dCK activity decreases so may dNTP and possibly NTP pools do as well.

In order to determine if the electroporation itself or if any of the siRNAs used could influence gene expression besides dCK downregulation, a whole-genome microarray experiment was conducted. Even though several genes were upregulated in the electroporated cells, these changes were rather modest and involved in systems likely to be affected during electroporation and were probably upregulated in order to help the cells cope with the electroporation process. This was not very surprising since electroporation is stressful to the cells, may damage their membranes, and sometimes lead to apoptosis, and the genes upregulated were most probably increased to help the cells repair their membrane damages, increase glucose uptake, or help the cells die if irreversibly damaged. When downregulating dCK, additionally two genes were affected and upregulated, they were however of unknown function. Three additional genes besides dCK, and involved in nucleoside analog metabolism, were evaluated using realtime-PCR. For dCK and TK2 there were a slightly higher difference between the microarray expression value and realtime-PCR expression value. This may be due to the fact that the array uses several housekeeping genes for normalization, while the realtime-PCR only normalized to one gene, GAPDH.

Conclusions

Based on the above results, this shows that suspension cells can be transiently transfected with siRNA to a rather high efficiency and specificity, and that siRNA is a powerful tool to study nucleoside analog metabolism. It also shows that the decreased sensitivity is seen against nucleoside analogs and not to anthracyclines, as is seen when making cells nucleoside analog-resistant using nucleoside analogs in the

cell medium during several months (Lotfi et al. 2002). We aim to use this technique further in the study of nucleoside analog resistance.

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Conflicts of interest None to declare.

References

- Arner ES, Eriksson S (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol Ther* 67:155–186
- Bahnsen AB, Boggs SS (1990) Addition of serum to electroporated cells enhances survival and transfection efficiency. *Biochem Biophys Res Commun* 171:752–757
- Bai L, Yamaguchi M, Tatsumi M, Kon K, Brautigam M (1998) Mechanisms responsible for resistance of sublines derived from leukemia cell lines to an antitumor agent 9-beta-D-arabinofuranosyl-2-fluoroadenine. *J Cancer Res Clin Oncol* 124:367–373
- Bergman AM, Eijk PP, van Ruiz Haperen VW, Smid K, Veerman G, Hubeek I, van den Ijssel P, Ylstra B, Peters GJ (2005) In vivo induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. *Cancer Res* 65:9510–9516
- Ferret E, Evrard C, Foucal A, Gervais P (2000) Volume changes of isolated human K562 leukemia cells induced by electric field pulses. *Biotechnol Bioeng* 67:520–528
- Fox M, Boyle JM, Kinsella AR (1991) Nucleoside salvage and resistance to antimetabolite anticancer agents. *Br J Cancer* 64:428–436
- Fyrberg A, Albertioni F, Lotfi K (2007) Cell cycle effect on the activity of deoxynucleoside analogue metabolising enzymes. *Biochem Biophys Res Commun* 357:847–853
- Goan YG, Zhou B, Hu E, Mi S, Yen Y (1999) Overexpression of ribonucleotide reductase as a mechanism of resistance to 2, 2-difluorodeoxycytidine in the human KB cancer cell line. *Cancer Res* 59:4204–4207
- Guittet O, Hakansson P, Voevodskaya N, Fridt S, Graslund A, Arakawa H, Nakamura Y, Thelander L (2001) Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *J Biol Chem* 276:40647–40651
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4:249–264
- Lembo D, Griboaud G, Hofer A, Riera L, Cornaglia M, Mondo A, Angeretti A, Gariglio M, Thelander L, Landolfo S (2000) Expression of an altered ribonucleotide reductase activity associated with the replication of murine cytomegalovirus in quiescent fibroblasts. *J Virol* 74:11557–11565
- Liu Y, Bergan R (2001) Improved intracellular delivery of oligonucleotides by square wave electroporation. *Antisense Nucleic Acid Drug Dev* 11:7–14
- Lotfi K, Mansson E, Spasokoukotskaja T, Pettersson B, Lillemark J, Peterson C, Eriksson S, Albertioni F (1999) Biochemical pharmacology and resistance to 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, a novel analogue of cladribine in human leukemic cells. *Clin Cancer Res* 5:2438–2444
- Lotfi K, Mansson E, Peterson C, Eriksson S, Albertioni F (2002) Low level of mitochondrial deoxyguanosine kinase is the dominant factor in acquired resistance to 9-beta-D-arabinofuranosylguanine cytotoxicity. *Biochem Biophys Res Commun* 293:1489–1496
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Mansson E, Flordal E, Lillemark J, Spasokoukotskaja T, Elford H, Lagercrantz S, Eriksson S, Albertioni F (2003) Down-regulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance. *Biochem Pharmacol* 65:237–247
- Meaking WS, Edgerton J, Wharton CW, Meldrum RA (1995) Electroporation-induced damage in mammalian cell DNA. *Biochim Biophys Acta* 1264:357–362
- Meldrum RA, Meaking WS, Wharton CW (1994) The kinetics and mechanism of repair of UV induced DNA damage in mammalian cells. The use of 'caged' nucleotides and electroporation to study short time course events in DNA repair. *Nucleic Acids Res* 22:1234–1241
- Melkonyan H, Sorg C, Klempt M (1996) Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO). *Nucleic Acids Res* 24:4356–4357
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Nordlund P, Reichard P (2006) Ribonucleotide reductases. *Annu Rev Biochem* 75:681–706
- Plunkett W, Grindey GB (1987) Pharmacokinetics of the 5'-triphosphates of arabinosylcytosine and 2',2'-difluorodeoxycytidine in L1210 cells. *Nucleic Acids Symp Ser* 77–79
- Verma NK, Dey CS (2004) RNA-mediated gene silencing: mechanisms and its therapeutic applications. *J Clin Pharm Ther* 29:395–404
- Wang X, Zhenchuk A, Wiman KG, Albertioni F (2009) Regulation of p53R2 and its role as potential target for cancer therapy. *Cancer Lett* 276:1–7
- Xie KC, Plunkett W (1996) Deoxynucleotide pool depletion and sustained inhibition of ribonucleotide reductase and DNA synthesis after treatment of human lymphoblastoid cells with 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl) adenine. *Cancer Res* 56:3030–3037
- Yu ZW, Quinn PJ (1994) Dimethyl sulphoxide: a review of its applications in cell biology. *Biosci Rep* 14:259–281
- Zhu C, Johansson M, Karlsson A (2000) Differential incorporation of 1-beta-D-arabinofuranosylcytosine and 9-beta-D-arabinofuranosylguanine into nuclear and mitochondrial DNA. *FEBS Lett* 474:129–132