

## ORIGINAL ARTICLE

# EFFECT OF CHLOROQUINE ON TRANSDUCTION OF CELL LINES AND BABOON CD34 CELLS BY A GALV PSEUDOTYPED RETROVIRUS

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**Background** – Chloroquine is an amine that inhibits lysosomal enzymes and is increasingly being used for increasing the rate of transfection of cells during gene transfer. Its effects on retrovirus-mediated gene transfer (transduction) are not clearly evident. Here, we studied the effects of this drug on transduction efficiency in a variety of cell lines and in hematopoietic cells.

**Methods** – We used a GALV pseudotyped retrovirus (PG13/ MNDEGFPSN) for transduction of the cell lines Hela, 208F, K562 and HL60, and baboon column-bead selected CD34+ cells using different methods, and in the presence of different concentrations of chloroquine. Transduction was gauged by detection of cells producing green fluorescence in a flow cytometer.

**Results** – Chloroquine (100  $\mu\text{mol}$ ) reduced transduction efficiency in cell lines, with some concomitant increase in propidium iodide positivity, which is an indicator of toxicity. This reduction in transduction efficiency remained in the generations of cells, even after the disappearance of conditions causing cellular toxicity. In baboon CD34+ cells, its effects were variable, but it did not increase the cellular toxicity. Increased transduction efficiency was observed in the presence of chloroquine (13.02  $\pm$  0.5% in the absence of chloroquine versus that in the presence of 50  $\mu\text{mol}$  (28.84  $\pm$  2.71%) and 100  $\mu\text{mol}$  (28.76  $\pm$  2.12%) of chloroquine). In other experiments, chloroquine increased PI positivity of the baboon cells and decreased transduction efficiency (19.09  $\pm$  0.61% and 17.99  $\pm$  0.22% without chloroquine in second and third experiments, respectively).

**Conclusion** – Although chloroquine inhibited transduction efficiency in cell lines, it had the opposite effect on baboon CD34+ cells and may actually increased transduction to these cells.

**Keywords** • chloroquine • GALV pseudotyped retrovirus • retroviral transduction  
• stem cells

## Introduction

Chloroquine is an amine that is widely used for increasing transfection of genes into cells.<sup>1</sup> It is a weak base with different effects on cellular physiology<sup>2</sup> and entry of enveloped RNA viruses into cells.

As a weak base, it rapidly enters cells within seconds, accumulating inside the acidic parts of the cell (including lysosomes and endosomes).

Because of this accumulation inside the cells,

some vacuoles appear. Accumulation of this weak base inside the cells leads to inhibition of lysosomal enzymes that require an acidic pH (~ 4 – 5) and also prevents fusion of endosomes and lysosomes, thereby preventing the degradation of ingested proteins and related foreign materials.<sup>2-4</sup>

Chloroquine also exerts toxic effects on cells that are linearly related to the concentration of the drug and the duration of exposure. It also prevents cellular protein synthesis and degrades certain cellular enzymes.<sup>3-5</sup>

Entry of enveloped RNA viruses (including retroviruses) into cells can be pH-dependent or pH-independent. For pH-dependent viral entry, the

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**Table 1.** Toxicity of different chloroquine concentrations during 48 hours of cell culture of 50,000 cells/well and exposure to drug.

	6 hours	12 hours	24 hours	48 hours
<b>208F cells</b>				
No drug				165,000
50 $\mu$ mol	ND	ND	40,000	
100 $\mu$ mol	ND	30,000	0	
200 $\mu$ mol	ND	0	0	
<b>Hela cells</b>				
No drug				500,000
100 $\mu$ mol	520,000	520,000		
<b>Baboon CD34+ cells (7 day culture in presence of growth factors)</b>				
No drug				1,850,000
50 $\mu$ mol	ND	1,500,000	ND	
100 $\mu$ mol	ND	Variable	0	
			From severe toxicity to > 300,000	

Values are the mean number of the alive cell in each wells; ND = not done; 0 = all of the cells died.

virus attaches itself to the receptor on the surface of the cell and is engulfed inside an endosome. Following reduction of the endosome, conformational changes occur in the viral receptor. This is followed by fusion of the envelope and endosomal surface, aiding the entry of the virion particle into the cytosol. Amines and other substances that prevent acidification of the endosome prevent both the exit of the original virus and entry of a new viral particle.<sup>6-9</sup>

For pH-independent viral entry, induction of conformational changes in the virus receptor are not required. The virus enters the cell directly from the cell surface, without any need for endosomal engulfment, or enters the cell so rapidly after engulfment that any associated pH changes are unnecessary. Amines (ammonium chloride, amantadine and chloroquine) cannot prevent entry of these viruses into cells.<sup>10,11</sup>

Most of the mammalian retroviruses use a pH-independent mechanism,<sup>10</sup> but some, such as ecotropic murine Moloney retrovirus, enter most cells as pH-dependent particles. However, in certain cell lines, it enters via a pH-independent mechanism.<sup>10</sup> The mechanism of entry is dependent on both the virus and the cell type.

In most studies, chloroquine has been used as an inhibitor of virus entry—a tool to determine the mechanism of virus entry. In some studies, it was successfully used to augment the transduction of retrovirus-based vectors. Hence, many researchers concluded that chloroquine increased transduction efficiency by prevention of lysosomal digestion of retrovirus inside cells, thus rescuing some of the viral particles, thereby increasing transduction efficiency.<sup>12</sup>

Chloroquine has other effects on cells, which include inhibition of synthesis of proteases, phospholipases and steroids, basal DNA synthesis and alteration of membrane fluidity. Also, it binds to the cell surface and membrane phospholipids,<sup>4</sup> and is toxic to several cell types.

In this study, the effect of chloroquine on transduction and associated toxicity of baboon CD34 cells and a variety of cell lines was studied.

## Materials and Methods

### Cell lines

To study the effects of chloroquine on retroviral transduction of cell lines, three cell lines were chosen: Hela cells, D17 and 208 F cells. K562 and HL60 cell lines and CD34-selected baboon BM hematopoietic cells without *in vivo* prestimulation were selected to study the effects of chloroquine on transduction of hematopoietic cells.

### Cell culture

For culture of Hela, D17 and 208F cells, we transferred 50,000 cells/well, to 6-well tissue culture dishes treated in the presence of DMEM containing 10% high fetal bovine serum (FBS) and 1% penicillin/streptomycin, 24 hours before transduction. For culture of K562 and HL60 cells, we transferred 50,000 cells/well to 12-well tissue culture dishes treated in the presence of 2 mL of RPMI containing 10% HIFBS and 1% penicillin/streptomycin and 50 ng/mL G-CSF into some of the wells to accelerate cell proliferation.

### Vector

We used PG13 packaging cell line containing a

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**Table 2.** Toxicity to the cells as determined by propidium staining.

	3 hours	6 hours	9 hours	12 hours
<b>Hela cells</b>				
No drug	0.96%			0.85%
100 µmol		0.96%	1.41%	1.48%
<b>208F</b>				
No drug				2.03%
100 µmol			2.35%	2.21%
<b>K562</b>				
No drug (without G-CSF)				18%
No drug (with G-CSF)				22.31%
100 µmol (without G-CSF)				27.72%
100 µmol (with G-CSF)				54.69%
<b>HL60</b>				
No drug (without G-CSF)				37.95%
No drug (with G-CSF)				31.51%
100 µmol ( without G-CSF)				71.14%
100 µmol (with G-CSF)				50.03%
<b>Baboon CD34+ 1<sup>st</sup></b>				
No drug				33.16%
50 µmol				22.71%
100 µmol				24.04%
<b>2<sup>nd</sup></b>				
No drug				14.74%
50 µmol				14.51%
100 µmol				25.94%
<b>3<sup>rd</sup></b>				
No drug				27.64%
50 µmol				32.56%
100 µmol				49.23%

G-CSF= granulocyt-colony stimulating factor.

GALV pseudotyped (gibbon ape leukemia virus) MNDEGFPSN vector (H.P. Kiem Lab, Fred Hutchinson Cancer Research Center, Seattle, WA, USA) to study expression of the GFP (green fluorescence protein), 48 hours after transduction. For production of vector after transfer of  $5.5 \times 10^6$  to 15-cm dishes, on day 3 and after subconfluency of the cell cultures, the medium was changed with a minimum of applicable medium (15 mL), and the vector was harvested, 12 hours later, for a total of three times. After filtering, the vector preparations were transferred to a  $-70^\circ$  freezer and used within

2 months after harvesting. The multiplicity of infection (MOI) from these harvests was about 3.

### Transduction

Cell line transductions were performed in the presence of 8 µg/mL of protamine sulfate with different concentrations of chloroquine (50 µmol – 100 µmol). Time of exposure to the drug ranged from about 6 – 12 hours. After transduction, the medium was changed after washing the wells with phosphate-buffered saline (PBS), and the cells were refed with DMEM or RPMI. Forty-eight

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**Table 3.** Transduction efficiency of cell lines.

		6 hours exposure and transduction	12 hours exposure and transduction
<b>Hela cells</b>			
	No drug	25.02%	28.27%
	100µmol	15.33%	6.42%
<b>208F</b>			
	No drug		15.25%
	100µmol		10.25%
<b>K562</b>			
	No drug		8%
	100µmol		5.9%
<b>(In presence or absence of G-CSF)</b>			
<b>HL-60</b>			
	No drug		< 2%
	100µmol		< 2%
<b>(in presence or absence of G-CSF)</b>			

hours after transduction, the expression of GFP was read by a flow activated cell sorter (FACS) machine. In some of the samples, chloroquine was added 3 hours after beginning transduction for discrimination between toxicity or entry block, and some Hela cells were also transduced in the presence or absence of chloroquine at pH 5.8, to study the effects of pH changes on transduction. We prepared DMEM pH 5.0 by the addition of HCL, and then added 1 mL of the acidic medium to a subset of the wells that were untreated or pretreated with chloroquine for 15 minutes. After addition of an equal volume of vector containing normal pH DMEM, the final pH reached 5.8. After exposure of the cells to these conditions, the medium was changed by adding fresh normal pH DMEM. We repeated transduction studies on cell lines three times for each cell line, and each study was repeated at least once. All of the values obtained are shown as the mean of the transduction efficiencies (the duplicated results of the range in each experience were very close). However, we are not reporting results of the entire range for simplicity of reporting.

### Toxicity study

To study cell toxicity, 50,000 cells/ well of 208F and Hela cells were transferred to 6-well

dishes and after 24-hours of transfer we changed to medium containing chloroquine (50 and 100 µmol). Cells were exposed to this concentration of the drug for 6 – 24 hours. After 48 hours and after trypsinization of the cells and trypan blue exclusion, the cells were counted and compared with the number of cells in the untreated wells. Further, on FACS analysis, we stained the cells with propidium iodide to exclude severely injured cells from the study.

### Transduction of baboon CD34+ cells

After receiving fresh bone marrow CD34+ cells, which were sorted by a column bead method, cells were transferred (50,000 cells/well) to 12-well non-tissue culture dishes, that were coated with recombinant fibronectin fragment (CH-296, 2 µg/cm<sup>2</sup>). Cells were prestimulated for 24 hours with a cocktail of megakaryocyte growth and development factor (MGDF) (50 ng/mL), human stem cell factor (50 ng/mL), interleukin-6 (50 ng/mL) and FLT-3 ligand (50 ng/mL). Such concentrations were sustained throughout the transduction and the results were recorded. 24 hours after prestimulation, the cells were transduced, once or four times, every 12 hours, in the presence of 8 µg/mL of protamine sulfate.<sup>13</sup> Between 50 and 100 µmol chloroquine was used

**Table 4.** Effects of addition of chloroquine, 3 hours after beginning of transduction.

Chloroquine	6 hours transduction administration of chloroquine after first 3 hours	12 hours transduction administration of chloroquine after first 3 hours
<b>Hela cells</b>		
No drug	25.02%	28.27%
100µmol	17.2%	9.6%

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**Table 5.** Flow-activated cell sorting results—days after transduction of Hela cells with chloroquine added at the onset of transduction, or 3 hours later.

	6 hours transduction		12 hours transduction	
	Concomitant	3 hours later	Concomitant	3 hours later
No drug	13.27%		16.36%	
100 $\mu$ mol	8.4%	5.7%	3.8%	3.7%

during the last transduction (for single transduced wells during transduction). Results were recorded for expression of GFP, 7 days after beginning prestimulation.

### FACS analysis

Transduction results were read by FACS analysis, after trypsinization and staining of the cells with 2  $\mu$ g/mL PI for fluorescein isothiocyanate (FITC) and PI, and analyzed the data after exclusion of dead cells. The number of cells studied numbered at least 10,000 in each reading.

## Results

### Toxicity study

Results of the toxicity study by trypan blue exclusion of Hela cells and 208F cells are shown in Table 1. For baboon CD34+ cells, in one experiment with good transduction efficiency, no chloroquine-associated toxicity appeared, probably because toxicity of chloroquine is concentration-dependent and exposure time-dependent, and each is different for various cell types. Also, chloroquine caused some changes in the shape of the cells, which led to forward and sidescatter during FACS analysis, which is more evident at higher concentrations of the drug.

Chloroquine toxicity to the cells was also studied by comparing the percentage of PI-positive cells at the time of FACS analysis (Table 2). Thus, it seems that although chloroquine is toxic to hematopoietic cell lines at concentrations higher than 50  $\mu$ mol, it is tolerable in these cell lines at lower concentrations.

### Transduction efficiency of cell lines

Several different cell lines were chosen for transduction to understand mechanisms of action

**Table 6.** Effect of pH change (for 6 hours) on transduction efficiency of Hela cells.

	Normal pH	pH 5.8
No drug	25.02%	25.49%
100 $\mu$ mol	15.33%	16.86%

of chloroquine. Transduction efficiencies differed in different cell lines in response to different concentrations of chloroquine and different exposure times to the drug (Table 3). It is clear that the chloroquine-associated reduction in transduction efficiency was proportional to the time of exposure to drug. To further explore this effect, chloroquine was added to cells 3 hours after transduction. The later addition of the drug improved transduction efficiency, but did not correct it completely (Table 4).

The question remains, is the effect of chloroquine due to the toxicity of drug on gene expression (it affects protein synthesis in the cells) or does it affect entry of the virus into the cell, or viral replication (reverse transcription) and integration of the vector?

To answer this question, transduced cells were cultured for 4 days (instead of the usual 2 days) before reading the FACS results to eliminate temporary toxicity to the cells. In this experiment, another subculture of the Hela cells was used, in which the transduction efficiency was lower than in previously described experiments (Table 5). Also, Hela cells were transduced with pH 5.8 growth medium, in the presence or absence of chloroquine added before transduction, to discriminate between pH-induced vector entry block from other possible effects (Table 6). The change in pH had no effect on transduction efficiency under these conditions.

### Transduction of baboon CD34+ cells

Baboon CD34+ cells tolerated different concentrations of chloroquine for less than 12 hours of exposure, but longer periods caused all of the cells to die. Transduction efficiency varied in different experiments (Table 7) and although, chloroquine increased transduction in one experiment, it did not increase it in another. In these experiments, we transduced column-selected baboon bone marrow CD34+ cells four times. The transduction efficiencies without chloroquine were similar in three experiments and although, addition of 50  $\mu$ mol chloroquine had no major effects in the second and third experiments, it increased

**Table 7.** Effect of chloroquine on transduction of CD34+ cells.

Experiment	Without chloroquine	50 $\mu$ mol	100 $\mu$ mol
1	13.02 $\pm$ 0.5%	28.84 $\pm$ 2.71%	28.76 $\pm$ 2.12%
2	19.09 $\pm$ 0.61%	18.25 $\pm$ 0.41%	9.31 $\pm$ 1.5%
3	17.99 $\pm$ 0.22%	16.33 $\pm$ 4.28%	9.25 $\pm$ 2.3%

transduction efficiency in the first experiment. Similarly, chloroquine at 100  $\mu$ mol reduced transduction efficiency in the second and third experiments and increased it in the first one. Thus, it was concluded that chloroquine at 50  $\mu$ mol had no negative effect on transduction of baboon CD34+ cells. In the second experiment, CD34+ cells were transduced by one exposure to the vector only, with and without chloroquine. Chloroquine at 50  $\mu$ mol had a small negative effect on transduction efficiency (11.74  $\pm$  0.8 without chloroquine vs 7.08  $\pm$  0.6 at 50  $\mu$ mol chloroquine). The higher chloroquine concentration (100  $\mu$ mol) prevented transduction profoundly (1.31  $\pm$  0.5).

The population of the cells in the three studies differed from each other based on lineage negativity and the number of the CD34-cell contamination was higher in the third experiment (Table 8).

The different responses to chloroquine observed during our experiments on baboon CD34+ cells may be due to different proportions of lin-positive cells (76.8% in sample 1, 24.71% in sample 2 ; undetermined for sample 3) and CD34+ cells (92.13% in sample 1, 94.63 % in sample 2 and 79.64% in sample 3).

Also, the proportion of mature large cells (according to forescatter and sidescatter characteristic of the cells) in these three experiments differed after 7 days of culture (15.38%, 12.93% and 27.85% in samples 1 – 3, respectively).

## Discussion

What are the exact biologic effects of chloroquine during transduction of cells with retroviral vectors?

According to our study, it seems that chloroquine affects transduction of the cells in the initial phase of transduction rather than last phase—probably before integration of the virus—because even after culturing the cells for a long period of time to circumvent the toxic effects of the drug on the transduced cells and their progeny, there was a reduction in transduction efficiency. Also, it seems that this effect is related to exposure time to the drug and its concentration. It seems that its inhibitory effects of drug on transduction related to initial first steps of viral entry to the cells, before integration of the virus. Although the pH-dependency or independency of the virus at entry was not demonstrated, and inhibition of transduction did not resolve with late addition of chloroquine so these steps are latter events in biology of retroviral genome integration. In another experiment not described here, when Hela cells were continuously transduced twice for 12 hours and 100  $\mu$ mol of chloroquine was added in the second transduction, transduction efficiency was halved in comparison to the control group not treated with chloroquine at all ( 43.1% vs 23.6%).

The effects of chloroquine on CD34+ cells were more complex than other cell lines. This

**Table 8.** Differences in composition of baboon CD34+ cell populations.

Experiment	CD34+ lin-positive	CD34+ lin-negative	No drug	50 $\mu$ mol	100 $\mu$ mol
1	Lin-positive CD34+ High FSC/SSC cells	76.8% 92.13%	20.7 $\pm$ 0.3%	24.48 $\pm$ 0.4%	29.34 $\pm$ 0.2%
2	Lin-positive CD34+ High FSC/SSC cells	24.7% 94.63%	16.77 $\pm$ 1.2%	15.35 $\pm$ 0.8%	13.9 $\pm$ 0.3%
3	Lin-positive CD34+ High FSC/SSC cells	Not done 79.64%	28.9 $\pm$ 0.7%	27.6 $\pm$ 0.5%	35.4 $\pm$ 5.1%

FSC = Forescatter, SSC = Sidescatter.

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complexity may have been due to heterogeneity of the cell populations or a more complex process of transduction of the cells.

Also we know that chloroquine is extruded from cells by a multiple drug resistance protein (MRP) gene, so it may be possible that the concentration of this drug that is achieved inside of CD34<sup>+</sup> cells is much lower than other cell lines.<sup>14</sup>

This study was limited by the proportion of more mature cells after transduction and also the amount of CD34 cells.

Further, the amount of PI-positive cells in these three studies was variable, and increased after chloroquine exposure. Although PI positivity in nontransduced cells varied among cells, the response of these cells to chloroquine was opposite to their response to transduction, so when chloroquine induced PI positivity, it decreased transduction efficiency and vice versa. So it may be the lower concentration of chloroquine or its use in MRP-positive cells that increases transduction, by inhibition of lysosomal enzymes and prevention of intracellular destruction of the viral particles by these enzymes.

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