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Research Article

Chloroquine Enhances Expression of Lipofected ivt-mRNA

Marina Tusup[1,2], Lars E French[1,2], Emmanuella Guenova[1,2], Thomas Kundig[1,2], and Steve Pascolo[1,2]*

[1]*Department of Dermatology, University Hospital of Zürich, Switzerland*
[2]*Department of Medicine, University of Zurich, Zurich, Switzerland*

***Corresponding author**
Steve Pascolo, University Hospital of Zürich; Department of Dermatology, Gloriastrasse 31, 8091 Zürich, Switzerland, Email: steve.pascolo@usz.ch

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Abstract

The transient expression of a protein *in vitro* or *in vivo* can be obtained by transfection of the *in vitro* transcribed messenger RNA (ivt mRNA). Delivery of ivt mRNA for therapies is typically based on liposomal formulations. The trapping and possible metabolic degradation of the liposome's payload within acidic/digestive compartments can limit the efficacy of this transfection method. By raising the pH in acidic vesicles, chloroquine inhibits endosome and lysosome activities. Thus, we tested whether chloroquine can enhance the expression of ivt mRNA delivered by lipofection. Using luciferase coding ivt mRNA, we found that at an optimal dose and schedule, chloroquine could increase the amount of protein produced from ivt mRNA formulated in liposomes. This method could be used as a generic way to potentiate the efficacy of therapeutic ivt mRNA formulations in development.

INTRODUCTION

Gene therapies based on the utilisation of *in vitro*-transcribed messenger RNA (ivt mRNA) are currently the focus of intense investigations [1-4]. This format has several advantages: it cannot integrate in the genome and is transient, thus guaranteeing safety. Independent of its sequence, it can be easily produced under good manufacturing practice (GMP) and stored [5]. Thus, it could be brought to clinical evaluation [6-9], rapidly after the demonstration of efficacy in pre-clinical models [10,11]. Although ivt-mRNA-based vaccines can be administered as naked molecules in isotonic buffers [12], for vaccination, high expression levels needed for gene complementation (e.g., cystic fibrosis, alpha-1-antitrypsin deficiency) [13], or expression of therapeutic proteins (e.g., erythropoietin [14], antibodies [15,16]) require packaging of the nucleic acid in efficacious transfection formulations. Cationic liposomes are widely used to deliver ivt mRNA. Such lipid-based nanoparticles exploit a variety of internalisation pathways for entering a cell, including clathrin- and caveolae-dependent endocytosis, and macropinocytosis [17]. Entry mechanisms may depend on the liposome characteristics and cell type. However, they usually result in clustering of the liposomes in endosomal compartments. A concomitant destabilisation of lipoplex and endosomal membranes can result in the release of the nucleic acid in the cytosol. Chloroquine, being a lysosomotropic agent, was reported to increase the release of siRNA from liposomes to cytosol [18,19]. By raising the pH in endosomes, chloroquine has a plethora of biological effects [20]. This drug and its derivatives, such as hydroxychloroquine, are used clinically to prevent or cure malaria and to treat autoimmune disorders as well as cancer [21]. Some mechanisms underlying those clinical effects are known, but new functions of the drug are regularly discovered. In the context of the adjuvant effect of chloroquine on chemotherapy, we reported that it depends on the dose and relative administration schedule of the drugs [22,23]. Keeping in mind the dose- and schedule-dependent effect of chloroquine on cellular and physiological pathways, we tested the effect of this drug on the expression of ivt mRNA formulated in liposomes. Our results show that at a precise schedule and dosage, chloroquine is capable of enhancing the efficacy of ivt-mRNA-based interventions.

MATERIALS AND METHODS

Production of ivt mRNA

The academic ivt-mRNA-production-and-formulation-platform in Zurich (http://www.cancer.uzh.ch/en/Research/mRNA-Platform.html) provided mRNA coding for firefly luciferase and ZsGreen [24]. The 5' end consisted of a CleanCap™ (Trilink) and an eIF4G-binding aptamer [25]. The coding sequence was codon optimised for use in mammalian cells. The 3' end consisted of a tandem repeat of the mouse beta-globin 3'UTR [24,26], and a poly-A tail. Uridine residues were fully substituted by 1-methyl pseudouridine residues, as this modification alone is enough to prevent immunostimulation while being efficiently translated [24].

Cells and transfection

The CT26 mouse colon carcinoma cells were maintained in a RPMI medium (Thermo Fisher Scientific) containing 10% foetal calf serum (FCS) and 0.2% antimicrobial reagent Normocin (InvivoGen). Transfection was performed by adding a mixture of 100 ng of mRNA in 2.5 microlitres of Opti-MEM (Thermo Fisher Scientific) and 200 ng of the lipofectamine transfection reagent MessengerMAX (Thermo Fisher Scientific) in 2.5 microlitres of Opti-MEM to 100,000 tumour cells in 200 microlitres of the RPMI medium supplemented with 10% FCS and 0.2% of the antimicrobial reagent Normocin (Invivogen). Fluorescence (Excitation: 485 nm; Emission: 528 nm) was recorded in real time at 37°C using Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek).

Animals and in vivo imaging

Our study "Anti-cancer therapies based on RNA" was approved by the Veterinary Office and its research ethics review committee of the University of Zurich (Kanton Zürich, Health Direction, Veterinary Office, Zollstrasse 20; 8090 Zurich; license number ZH215/17). Animals were purchased from Envigo (Netherlands). 4- to 8-weeks-old mice were injected intravenously with 1 or 5 micrograms of mRNA formulated with a TransIT® transfection kit following the manufacturer's suggestions (Mirus). The procedure for 1 microgram of mRNA was as follows: the nucleic acid was diluted in 38 microlitres of Opti-MEM (Gibco) before the addition of 0.72 microlitres of the "mRNA Boost Reagent". After mixing, 1.12 microlitres of the TransIT reagent were added. The solution was thoroughly homogenated and injected immediately: 40 microlitres per mouse for 1 microgram of mRNA and 200 microliters per mouse for 5 micrograms of mRNA. Eventually, the mice received 50 micrograms of chloroquine (chloroquine phosphate from Sigma) injected in 200 microlitres intraperitoneally. At different time points after mRNA injection, in vivo bioluminescence imaging was performed on an IVIS Lumina instrument (PerkinElmer). Immediately before each measurement, D-luciferin (Synchem) dissolved in PBS (15 mg/ml stock) and sterile filtered was injected (150 µg/g intraperitoneally). Emitted photons from live animals were quantified 10-20 minutes post luciferin injections, with an exposure time of 3 min. Regions of interest (ROI) were quantified for average radiance (photons s−1 cm−2 sr−1) (IVIS Living Image 3.2).

RESULTS AND DISCUSSION

Dose- and schedule-dependent effect of chloroquine on ivt mRNA lipofection

Chloroquine can potentially affect cell biology in many ways; therefore, we tested its effect on lipofected ivt mRNA expression using cells (i) pre-treated one hour with the chloroquine, (ii) treated at the same time with chloroquine and the liposome or (iii) treated with chloroquine two hours after the start of incubation with the liposome. The concentration of chloroquine ranged from 0.15 µg/ml to 5 µg/ml, overlapping the range of clinical usage (2 mg per kg up to 5 mg per kg). Evaluating translation of the ZsGreen-coding ivt mRNA by monitoring fluorescence in the cultures (Figure 1), it appeared that the addition of chloroquine before or at the time of transfection did not enhance the expression of the ivt mRNA. In contrast, low doses of chloroquine (0.15 µg/ml up to 1.25 µg/ml with 0.3 µg/ml being best) added two hours after the transfection could result in an increased production of ZsGreen.

Administration of chloroquine increases expression of injected ivt mRNA formulated in liposomes

The intravenous injection of firefly luciferase-coding ivt mRNA formulated in the TransIT® delivery system allows detection of the encoded protein predominantly in the liver with a peak of expression ca. 4 hours [27]. When mice received a low dose of 50 micrograms (i.e., 2 mg/kg) of chloroquine two hours after intravenous injection of one microgram of ivt mRNA in TransIT®, expression of the encoded enzyme was twice as high than in mice not receiving chloroquine (Figure 2A): the mean radiance is 2.688×105 in the RNA alone versus 4.470×105 in the RNA-chloroquine combination. Injection of chloroquine at the time or prior RNA injection did not result in robust enhancement of ivt mRNA expression (data not shown). Following the kinetics

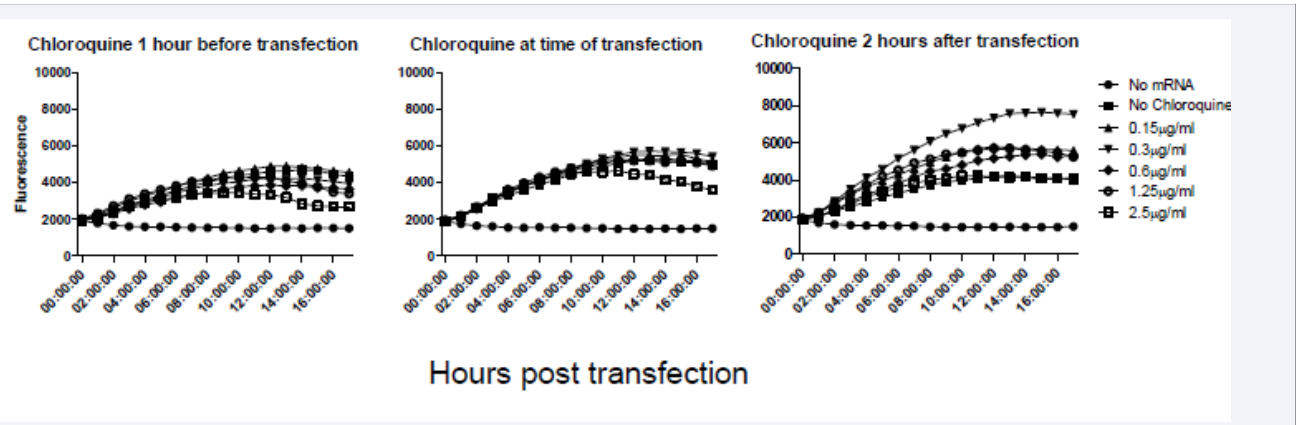

Figure 1 Effects of chloroquine on the expression of lipofected ivt mRNA *in vitro*. Synthetic mRNA coding ZsGreen was transfected using MessengerMAX in mouse CT26 cells. Chloroquine was added in the cell culture at different doses either one hour before (left panel), at the same time (middle panel) or two hours after (right panel) lipofection. Fluorescence developing in the cell cultures was monitored every hour. The data show that intermediate doses of chloroquine added 2 hours after lipofection can enhance the production of ZsGreen in transfected cells.

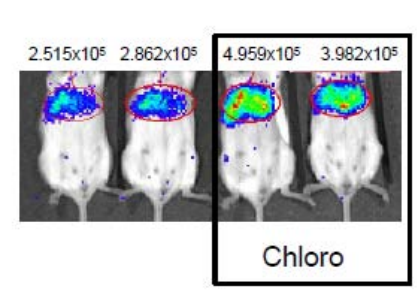

Figure 2a Effects of chloroquine on expression of lipofected ivt mRNA *in vivo*. Synthetic mRNA coding firefly luciferase was injected intravenously in a lipid-base formulation, allowing expression predominantly in liver. In A, all four mice received one microgram of ivt mRNA. In addition, two mice ("Chloro") received 50 micrograms of chloroquine two hours after injection of the mRNA. The luciferase activity was recorded *in vivo* 5 hours after the injection of RNA (the numbers indicate radiance in the region of interest). Red indicates high radiance, while blue indicates low radiance. The results demonstrate that the administration of Chloroquine can increase the production of luciferase from injected ivt mRNA formulated in TransIT®.

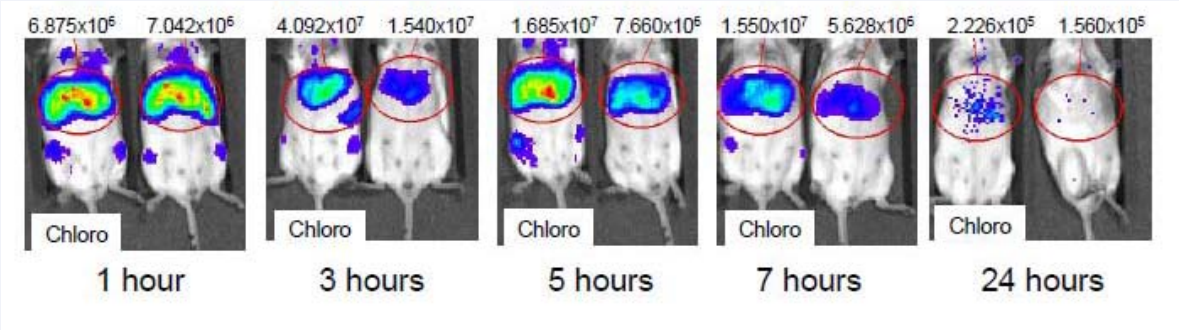

Figure 2b Effects of chloroquine on expression of lipofected ivt mRNA *in vivo*. Synthetic mRNA coding firefly luciferase was injected intravenously in a lipid-base formulation, allowing expression predominantly in liver. In B, two mice received five micrograms of ivt mRNA. In addition, the mouse on the right ("Chloro") received 50 micrograms of chloroquine two hours after injection of the mRNA. The luciferase activity was recorded *in vivo* 1 hour, 3 hours, 5 hours, 7 hours and 24 hours after the injection of ivt mRNA coding luciferase (the numbers indicate radiance in the region of interest). Red indicates high radiance, while blue indicates low radiance. The results indicate that although luciferase expression was similar in both mice at one hour, it increased in the mouse receiving chloroquine compared to the mouse not receiving chloroquine at later time points (3 hours and more).

of the expression (Figure 2B), it appeared that evidence for the sustained expression of luciferase can be observed one hour after the injection of chloroquine (3 hours after injection of ivt mRNA in TransIT®). Twenty-four hours after mRNA injection, a clear luciferase signal was detected in mice that received the mRNA-chloroquine combination, while barely any signal was detected in mice receiving only RNA as reported earlier [24].

CONCLUSION

We report here that the expression of formulated ivt mRNA can be increased by the adequate (time and dose) utilisation of chloroquine in vitro and in vivo. Chloroquine has many reported biological activities; therefore, it is difficult to pinpoint precisely the mechanism of action whereby this drug enhances expression of the protein encoded by ivt mRNA. We postulate that chloroquine given two hours after transfection may help in destabilising endosomes where the nanoparticles are located and thereby enhances the delivery of ivt mRNA to cytosol. However, we cannot exclude that other activating or inhibiting effects of chloroquine on the cells' biology (e.g., inhibition of autophagy) are at the origin of the effect we report. Intriguingly, as it is the case for chemotherapy [22,23], the effect of chloroquine is dose and schedule dependent. Administration of low doses of chloroquine after other drugs/formulations seems to be preferred. We foresee that the adequate utilisation of chloroquine might be a safe and cost-effective way to enhance the efficacy of ivt mRNA-based therapies.

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AUTHORSHIP CONTRIBUTIONS

MT performed experiments and participated in the revision of the manuscript.

TK supported the study and participated in the revision of the manuscript.

EG supported the study and participated in the revision of the manuscript.

LF supported the study and participated in the revision of the manuscript.

SP designed the study, performed experiments and wrote the manuscript.

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