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Schedule-Dependent Synergy of Chloroquine with Chemotherapy for Anti-Cancer Treatment

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Abstract:

Although it is a very safe compound, the antimalarial drug chloroquine raises the endosomal pH in all cells of an organism, thereby modifying fundamental cell functions and physiological systems. This impact may promote or antagonize the activity of other drugs such as anti-cancer chemotherapeutics. One published placebo-controlled clinical study indicated that chloroquine may enhance the efficacy of chemo- and radio-therapies. However, the optimal timing and dosing of chloroquine were not investigated. Because of the many potential effects of chloroquine on tumor cells, tumor microenvironment and immunity, we undertook an unbiased evaluation of the effect of chloroquine on the therapeutic efficacy of gemcitabine in tumor bearing mice *in vivo*. We surprisingly found that the antimalarial drug synergizes strongly with the anti-cancer chemotherapy only when it is administered after gemcitabine. *In vitro* studies indicated that the synergy is a direct consequence of chloroquine activity on tumor cells and is independent of autophagy. Importantly, the adaptive immune system is required for elimination of the tumor *in vivo*. Our results stress the high clinical anti-cancer potential of chloroquine as an adjuvant to chemotherapy and suggest that the therapeutic effect of this lysosomotropic drug requires a specific administration schedule within standards of care protocols.

Keywords: Chloroquine; Chemotherapy; Gemcitabine; CT26; Cancer

Introduction

Chloroquine (N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine), a chemical molecule related to the naturally occurring quinine from the bark of cinchona trees (review by Solomon, [1]), has been used to prevent (single dose at *ca.* 2 mg/kg once a week *i.e.*, one tablet of 100 mg for an adult) or cure (6 mg/kg daily over three consecutive days, *i.e.*, three tablets of 100 mg per day for an adult) malaria for more than sixty years. More recently, the anti-inflammatory potential of a high dose chloroquine regimen (6 mg/kg daily) has been introduced in the treatment of inflammatory diseases such as rheumatoid arthritis and lupus erythematosus. The drug diffuses freely and rapidly across cell membranes. Although very safe at prescribed dosages, chloroquine can cause dose-related retinopathy when administered for prolonged periods at more than 30 mg/kg/day. At low pH, such as in endosomes, chloroquine is protonated and trapped in organelles. As a weak base, its accumulation increases the pH of endosomes and lysosomes, thereby altering the activity of many enzymes. As a consequence, proteolysis and metabolism of neoglycolipids is hampered [2], catabolism of proteins and subsequent MHC antigen presentation is modified [3 - 5] and secretion of proteins, including inflammatory cytokines, through the exocytic pathway is decreased [6]. In selected tumor cell lines, chloroquine alone at a relatively high concentration can directly induce differentiation, necrosis and apoptosis (reviewed by Solomon [1]). Significant *in vivo* anti-cancer activities of chloroquine alone required dosages above safety limits in mice (review by Pascolo[7]). For example, using the colon carcinoma model CT26 in BALB/c mice, Zheng et al. [8] have shown that the minimal dose of chloroquine as a single reagent for controlling partial tumor growth and enhancing survival was more than 175 mg/kg (daily dose of more than 25 mg/kg). Thus, chloroquine alone cannot be validated as an anti-cancer drug. However, at an acceptable dose, it has several activities that suggest a potential to enhance the efficacy of standard anti-cancer treatments (radio- and chemo-therapies) (reviewed by Pascolo[7]). Evidence includes direct effects of the drug on tumor cells, such as (i) inhibition of drug efflux pumps [9], (ii) toxicity on cancer stem cells [10, 11] and (iii) initiation of cell death pathways [12, 8]. Chloroquine can also have indirect effects on anti-cancer chemotherapies. For example, the drug's buffer effect in the acidic extracellular tumor milieu as well as in intracellular vesicles can assist in the release of sequestered basic chemotherapeutic compounds that are protonated at low pH (*e.g.*, Doxorubicin), thereby increasing their anti-cancer activity [13]. Altogether, pre-clinical data indicate that chloroquine could be a good adjuvant to radio- and chemo-therapies. Following this rationale, the highest dose of chloroquine (300 mg/day, daily) was combined with chemo-therapy (Carmustine) and radio-therapy for the treatment of post-surgical tumor ablation in glioblastoma patients. This combination was found to be safe and efficacious [14, 15]. Forty one patients receiving the combination had a survival time of 25 ± 3.4 months, compared with 11.4 ± 1.3 months in control subjects (radio-chemotherapy alone, 82 patients) (p = .000). In order to further examine the utilization of chloroquine as an adjuvant in chemotherapeutic treatment, we evaluated it in combination with 2', 2'-difluorodeoxycytidine (gemcitabine). This cancer drug from the metabolite analogue family acts through (i) inhibition of the ribonucleotide reductase those results in a reduction of the dNTP pool and (ii) lethal incorporation into DNA in place of dCTP. Gemcitabine is an immunological death inducer [16]. Part of its anti-cancer activity is attributed to the fact that cells dying from gemcitabine stimulate innate immunity and trigger an anti-cancer adaptive immune response. Thus, gemcitabine's efficacy requires a healthy immune system and may be limited when used in combination with immunosuppressive treatment, such as high dose chloroquine. Gemcitabine is used worldwide as a single-agent therapy against advanced pancreatic carcinoma and cholangiocarcinoma. Because chloroquine has many activities that could synergize or antagonize Gemcitabine's action, we undertook an unbiased investigation of the effect of single injections of the antimalarial drug at varying dosages and schedules on the therapeutic activity of a single injection of gemcitabine in tumor bearing mice. Our results indicate that chloroquine can indeed strongly enhance the anti-cancer efficacy of gemcitabine but only when it is administered on a specific schedule.

Materials and Methods

Tumor Transplantation

BALB/c mice (wild type or nude) received 2 million CT26/NY-ESO-1 cells (CT26 colon carcinoma cells stably expressing NY-ESO-1, kindly provided by Professor Hiroyoshi Nishikawa [17]) sub-cutaneously at the base of the neck in a total volume of 200 microliters of PBS. Tumor size was recorded using a caliper every other day starting at day 9 post-tumor implantation.

Mouse Treatment

Our study "Adjuvant effect of Chloroquine on chemo radio-therapies" was approved by the veterinary office of Zurich (Kanton Zürich, Health direction, Veterinary Office, Zollstrasse 20; 8090 Zurich; license number 175/2011). The veterinary office of Zurich has a research ethics review committee that granted approval. All efforts were made to minimize suffering. Animals were purchased from Harlan (Netherland). Mice with a tumor of approximately 0.25 cm^2 at day 9 were treated by injection of 75 microliters (2 mg) of gemcitabine (Lilly or Actavis Switzerland) intraperitoneally.

Either one day before or one day after gemcitabine administration, 200 microliters of a freshly prepared

chloroquine solution containing either 200 micrograms or 40 micrograms of chloroquine in PBS was injected intraperitoneally. Thus the detailed experiment is: Single sub-cutaneous injection of tumor cells at day 0; single intraperitoneal injection of gemcitabine at day 9; single intraperitoneal injection of Chloroquine either one day before or one day after gemcitabine; tumor size measurement (longest dimension) using a caliper three times per week; single peripheral blood draw from the tongue (vena sublingualis) while the mice are ca. 15 minutes under Isoflurane anesthesia at approximately day 14. Experimental endpoints are tumor size and immune responses. To prepare the chloroquine solution, one tablet of chloroquine was diluted in 10 ml of water, resulting in a 10 mg/ml stock solution, which was further diluted to 1 mg/ml or 0.2 mg/ml using PBS. Injections were performed by experienced personnel without anesthesia as stress was very limited (only sub-cutaneous and intraperitoneal injections were performed). Mice were killed by treatment with increasing concentration of carbon dioxide. Death was subsequently confirmed by cervical dislocation according to good animal management/practice.

Survival Study

We used humane endpoints during the animal survival study. Mice are assessed every other day. In addition to tumor size, mice in experiments were controlled for the following parameters: integrity of the fur and free movements. A score sheet was made as follows

(activity is meant after moderate stimulation):

Score	0	1	2	3
Tumor size	0-3mm	4-6mm	7-10mm	>10mm
Fur	Normal	Rough	Hair loss	
Activity	Normal	Decreased	Inactive	Immobile
Weight loss				>15%

A total score of 3 or more leads to killing of the mouse (of note, recorded tumor size may exceed 1 cm since tumors may increase size dramatically within two days). Criteria for discontinuing an experiment: 1: Mice transplanted with tumor cells will be monitored and culled if tumour size reaches 10 x 10 millimetres, i.e. before the tumour is able to have any systemic effect. 2: general illness as assessed by study director/ animal care staff and according to the score sheet, 3: Ulceration or necrosis of the tumor. There have been no unexpected deaths and no adverse health effects during the experiments reported here.

Immunomonitoring

Cytotoxic T-cell monitoring was performed in the peripheral blood. Total cells were stained with mouse CD4-FITC, mouse CD8-APC and PE-labelled MHC tetramers comprising H2-D^d monomers loaded with the RGPESRLL peptide, the immunodominant NY-ESO-1 epitope in the H-2^d haplotype [17]. Cells were then acquired using a Cyan FACS (Beckman Coulter) and data were analyzed using Flowjo software (Treestar).

In vitro Proliferation Assay

CT26 cells were plated in 24-well plates at 50 000 cells per well in 1 ml of complete medium (RPMI with 10% Fetal Calf Serum, penicillin and streptomycin). One day later, gemcitabine was added to a final concentration of 0.05 μg/ml to 0.09 μg/ml. Cells were incubated for 4 hours at 37°C, followed by replacement of the medium with complete medium. One day later, chloroquine was added at the indicated concentrations. Three days after the addition of gemcitabine (two days after the addition of chloroquine), the medium was removed, the wells were washed with RPMI and 1 ml of PBS was added. Plates were placed in a -80°C freezer overnight. To evaluate cell proliferation in cultures, LDH content in plates left at room temperature for four hours to thaw was measured using the Promega Cytotox 96 kit. While thawing, cells release LDH. Relative survival was calculated using the experimental value minus background (PBS plus LDH buffer) divided by the LDH value obtained with untreated cells minus background (PBS plus LDH buffer).

LC3 Western Blotting for the Detection of Autophagy

In order to obtain protein extracts, cells were collected, washed twice in PBS, resuspended in ice-cold lysis buffer (1% NP-40 with complete protease inhibitor cocktail from Roche) and incubated on ice for 10 min. The resulting cell lysates were centrifuged at 20.000 g for 10 min at 4°C. For all samples, equal amounts of total protein extracts were boiled for 5 min in the presence of SDS-PAGE loading buffer NuPage (Life Technologies) with 1% β-mercaptoethanol. Protein extracts were resolved in 12.5% SDS-PAGE gels and transferred onto PVDF membranes (GE healthcare). For detection of specific protein bands, primary antibodies, HRP- conjugated secondary antibodies and the ECL Plus detection systems were used (Thermo Scientific). Anti-Atg8/LC3 (clone 5F10) was purchased from Nano tools, and mouse anti β-actin (clone AC-15) from Abcam. Quantification of protein levels by densitometry was performed on digitalized films using the ImageJ software.

Results and Discussion

Chloroquine can enhance the Anti-Cancer Efficacy of Gemcitabine

In BALB/c mice, a single injection of gemcitabine intraperitoneally can reduce the growth of established (approximately 0.25 cm^2) subcutaneous solid tumors (CT26 cells; Figure 1A). However, a cure is rarely seen when examining survival (Figure 1B). Injection of 200 micrograms of Chloroquine one day before the administration of gemcitabine has no or a slightly negative effect on the disease outcome (Figure 1). On the contrary, injection of chloroquine one day after injection of gemcitabine, especially at the low dose of 40 micrograms per mouse, results in a better therapeutic effect: delayed tumor growth (Figure 1A) and frequent cure (Figure 1B). Of note, because gemcitabine has an *in vivo* half-life of 42 to 94 minutes for short infusions (http://www.rxlist.com/gemzar-drug.htm), the chemotherapeutic drug is virtually cleared from the organism when Chloroquine is administered in our experiments. Therefore, the synergy between the sequential administration of gemcitabine and chloroquine is not due to the interaction of the two drugs but rather to their independent physiological effects.

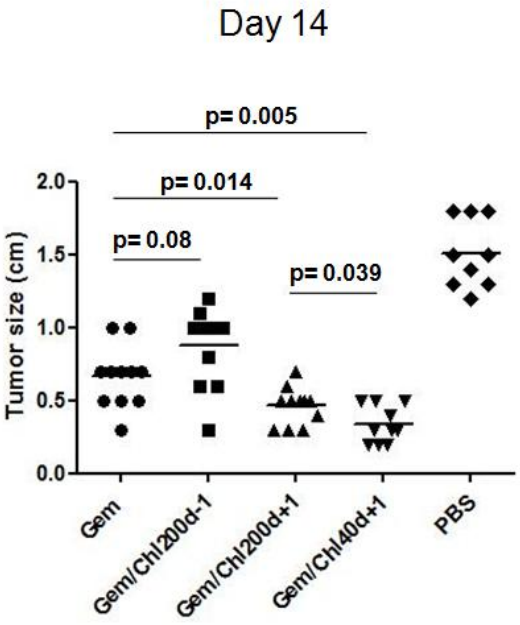

Figure 1A

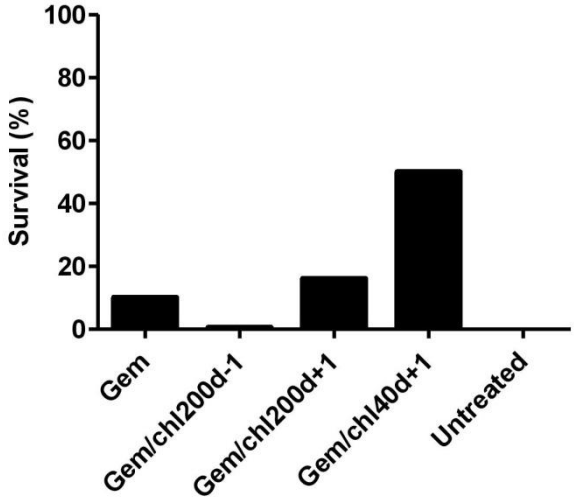

Figure 1B

Figure 1: Administration of chloroquine after gemcitabine delays tumor growth

At day nine post tumor implantation (CT26-NY-ESO-1), gemcitabine was injected intraperitoneally ("Gem"). Either one day before injection of gemcitabine ("Gem/Chl200d-1") or one day after ("Gem/Chl200d+1), 200 micrograms of chloroquine were injected. In addition, a group of mice received 40 micrograms of chloroquine one day after gemcitabine (Gem/Chl40d+1). Control mice only received PBS ("PBS"). Tumor size was recorded every other day using a caliper. In A, the graph represents tumor size on day 14 post-tumor implantation in one representative experiment. The experiment was repeated three times and similar results were obtained. In B, survival of mice indicated by the absence of tumor (no palpable tumor or residual sub-cutaneous lump of less than 0.25 cm^2) at day 30 post-tumor injection is presented. The graph is the sum of the total survival from three independent identical experiments.

The Gemcitabine-Chloroquine Combination has a Direct Cytotoxic Effect on Tumor Cells, Independent of Autophagy

To understand the anti-cancer synergy obtained when the two drugs are administered sequentially, we studied *in vitro* the impact of chloroquine on cancer cells pre-incubated with gemcitabine. As shown in Figure 2A, upper panel, treatment with a sub-optimal concentration of gemcitabine (0.05 or 0.07 μg/ml for 4 hours) that induces partial inhibition of cell growth, followed one day later by the addition of chloroquine at 2 mg/l, 4 mg/l or 6 mg/l (equivalent to standard *in vivo* dosages ranging from 100 mg to 300 mg for a humane adult) results in synergy. As previously shown [8], chloroquine alone in this dosage range does not induce cell death (Figure 2B). Thus, *in vitro* and in the absence of the tumor microenvironment or immune cells, gemcitabine and chloroquine have direct synergistic anti-proliferation activity on cancer cells. Autophagy is a mechanism that can be utilized by cells to survive a stress, such as a limitation of nutrients. By limiting the dNTP pool, gemcitabine creates the condition of metabolite deprivation. Indeed, analysis of the essential autophagic membrane attached protein LC3-II in CT26 cell lysates, which correlates with autophagosome accumulation [18], revealed after 4 hours of gemcitabine treatment gemcitabine-induced autophagy (Figure 2C). Of note, at least 0.07 μg/ml of gemcitabine seems to be required to induce a detectable LC3-II increase, while synergy between gemcitabine and chloroquine can be observed with a concentration of gemcitabine of 0.05μg/ml (Figure 2A). Autophagy-deficient cells stably expressing shRNA specific for ATG5 (one of the genes necessary for LC3 conjugation to autophagic membranes) are not more sensitive to gemcitabine than control CT26 cells (scrambled shRNA) and also display the anti-proliferation synergy between gemcitabine and chloroquine (Figure 2D). Thus, inhibition of autophagy by chloroquine (as exhibited with clinically unacceptable doses of chloroquine [19, 10, 20]) does not seem to be the mechanism underlying the synergy between gemcitabine and chloroquine.

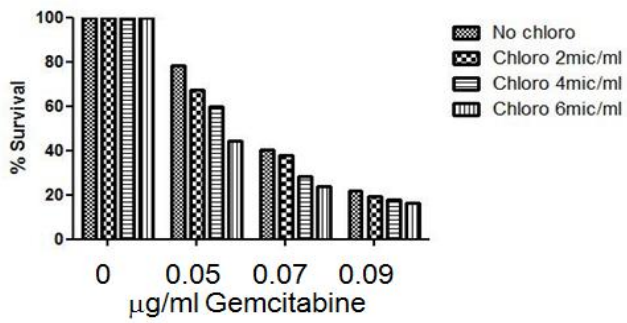

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Figure 2A

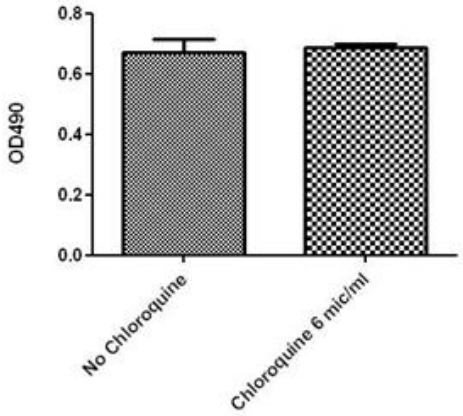

Figure 2B

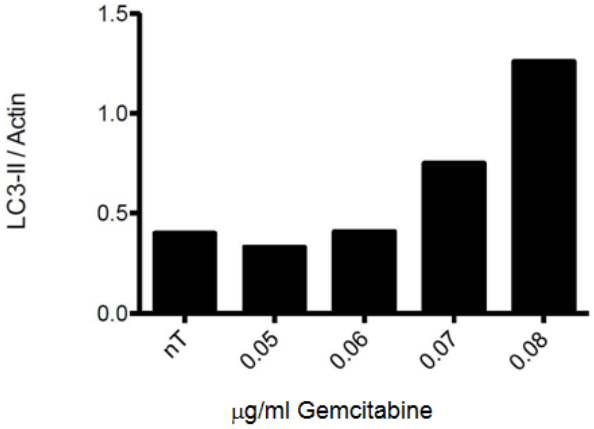

Figure 2C

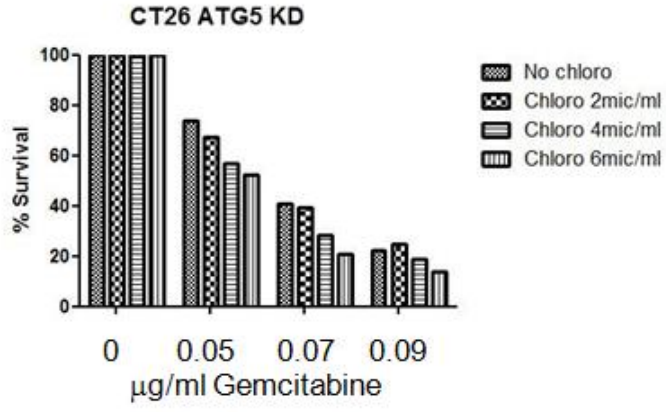

Figure 2D

Figure 2: Gemcitabine and chloroquine synergize *in vitro*

The survival of cells exposed to gemcitabine for 4 hours and then cultured in the presence of chloroquine was evaluated by LDH released by killed (frozen/thaw) cell cultures. In A, the graph represents survival of CT26 treated with different amounts of gemcitabine followed by varying amounts of chloroquine. In B, the survival of CT26 cells treated with chloroquine alone at 6 micrograms per ml is shown. The diagram in C depicts western blot analysis of LC3-II detected in cell lysates cultured after 8 hours (4 hours in the presence of gemcitabine followed by 4 hours in complete medium without gemcitabine) to determine the effect of gemcitabine on the induction of autophagy. The LC3-II bands are normalized to the β-actin control bands for each cell lysate. In D, the graph shows survival of autophagy-deficient CT26 cells, which express a shRNA that permanently down-regulates ATG5 (CT26 ATG5KD, kindly provided by Professor Laurence Zitvogel) [21].

Adaptive Immunity is required for the Efficacy of the Gemcitabine-Chloroquine Protocol

Certain chemotherapeutic drugs such as gemcitabine belong to a group of immunologic cell death inducers and contribute to the induction of anti-cancer immunity by dying tumor cells [16]. Importantly, the efficacy of gemcitabine is weak in T-cell deficient nude mice (Figure 3A). Although the use of chloroquine after gemcitabine resulted in a slightly improved outcome of the disease that was both statistically significant and reproducible, no cure was ever observed in those mice. Additionally, we found that a detectable *ex-vivo* cytotoxic immune response against NY-ESO-1 spontaneously developed in tumor-bearing wild type BALB/c mice and was not affected by treatment with gemcitabine alone or gemcitabine plus chloroquine treatment (Figure 3B).

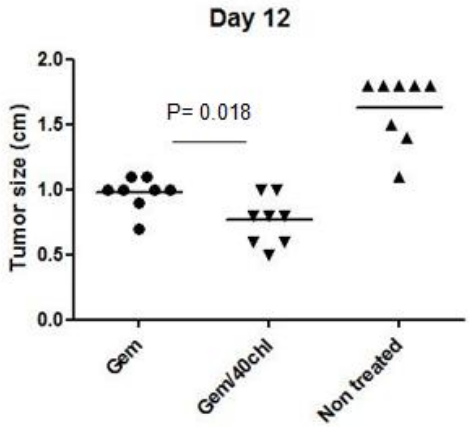

Figure 3A

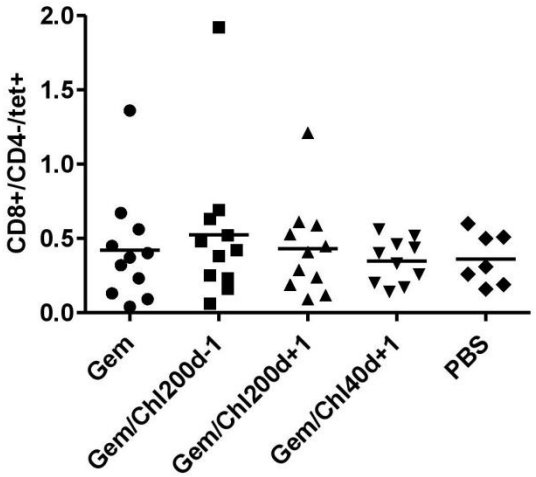

Figure 3B

Figure 3: Adaptive immunity is required for the efficacy of the gemcitabine/chloroquine treatment

The graph in A represents tumor size in nude BALB/c mice at day 12 post tumor implantation. Tumor bearing mice were treated with gemcitabine alone ("Gem") or with gemcitabine in combination with 40 micrograms of chloroquine injected one day later ("Gem/40Chl"). The graph in B represents the percentage of cytotoxic T-cells in the peripheral blood of CT26/NY-ESO-1 tumor bearing mice treated with gemcitabine alone ("Gem"), gemcitabine combined with 200 micrograms of chloroquine injected one day before ("Gem/Chl200d-1") or one day after ("Gem/Chl200d+1) or gemcitabine combined with 40 micrograms of chloroquine given one day later (Gem/Chl40d+1).

Conclusion

We conclude that optimal use of chloroquine as an adjuvant to gemcitabine occurs when chloroquine enhances gemcitabine-induced blockade of proliferation while not interfering with the induction of an anti-cancer immune response. Chloroquine may have several immunosuppressive features. For example, it may reduce the release of ATP by dying cells and compromise innate and subsequently adaptive immunity triggered by cells dying from gemcitabine. In addition, chloroquine may limit all necessary communication between immune cells by inhibiting the release of cytokines (i.e., communication between antigen presenting cells and T-cells or between helper T-cells and cytotoxic T-cells) thereby preventing the harmonious and complete development of anti-cancer immunity triggered by gemcitabine. The ability of chloroquine to inhibit immune function in these ways may account for the contra-productive effect of chloroquine when administered one day prior to gemcitabine. By contrast, the additional cryptic death-inducing effect of acceptable doses of chloroquine on tumor cells, such as the induction of vacuolation [12] at a time when the development of an innate (*e.g.*, release of molecules such as ATP by tumor cells dying from gemcitabine) and adaptive (release of cytokines by activated antigen presenting cells to attract and stimulate T-cells) immune response have been successfully initiated is beneficial. Alternatively or in addition, the toxicity of chloroquine on tumor stem cells that may have resisted gemcitabine and normally recolonized the tumor may account for the efficacy of the combination. The synergy is found particularly at a low dose of chloroquine, where further development of T-cells (activation and proliferation) that require the release of cytokines, is not affected. Although the dosage of chloroquine has a clear incidence on the efficacy of the combination, the administration schedule appears to be the most important parameter.

We hypothesize that enhanced cell death induced by chloroquine administered one day after gemcitabine in conditions (time/dose) where chloroquine does not limit innate or adaptive immune functions accounts for the efficacy of the gemcitabine-chloroquine combination and schedule described in this article. Based on these results, we have implemented a phase 1 dose escalation (100 mg up to 300 mg of chloroquine) clinical study for advanced pancreatic carcinoma patients eligible for gemcitabine monotherapy. We anticipate that the adequate (time/dose) uptake of chloroquine will be a safe, inexpensive and efficacious protocol that will result in a marked enhancement of gemcitabine's efficacy for the management of advanced pancreatic carcinoma and other diseases in which gemcitabine is injected alone or within a multidrug schedule, such as cholangiocarcinoma and lung cancer. Moreover, we foresee that the adjuvant effect of chloroquine could be extended to other chemotherapy drug/regimens and radiotherapy. Because chloroquine may be contra-indicated in some instances, (i.e., it should not be administered one day prior to gemcitabine), systemic dose/schedule pre-clinical studies as presented here must be performed to unravel, optimize and broaden the potential of Chloroquine as an adjuvant in cancer treatments.

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Author Contributions

IanaParvanova performed the *in vivo* experiments and immunological read out.

Alexander Knuth supported the study.

Heike Nowag and Christian Münz studied the induction of autophagy in tumor cells.

Steve Pascolo designed and supervised the study and performed *in vitro* and *in vivo* experiments.

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