Chloroquine targets pancreatic cancer stem cells via inhibition of CXCR4 and hedgehog signaling

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ABSTRACT

Pancreatic ductal adenocarcinoma is one of the deadliest carcinomas and is characterized by highly tumorigenic and metastatic cancer stem cells (CSCs). CSCs evade available therapies, which preferentially target highly proliferative and more differentiated progenies leaving behind CSCs as a putative source for disease relapse. Thus, to identify potentially more effective treatment regimens we screened established and new compounds for their ability to eliminate CSCs in primary pancreatic cancer (stem) cells in vitro and corresponding patient-derived pancreatic cancer tissue xenografts in vivo. Intriguingly, we found that in vitro treatment with the anti-malarial agent chloroquine significantly decreased CSCs translating into diminished in vivo tumorigenicity and invasiveness in a large panel of pancreatic cancers. In vivo treatment in combination with Gemcitabine was capable of more effectively eliminating established tumors and improved overall survival. The inhibitory effect of chloroquine was not related to inhibition of autophagy, but was due to inhibition of CXCL12/CXCR4 signaling resulting in reduced phosphorylation of ERK and STAT3. Furthermore, chloroquine showed potent inhibition of hedgehog signaling by decreasing the production of Smoothened, translating into a significant reduction in sonic hedgehog-induced chemotaxis and down-regulation of downstream targets in CSCs and the surrounding stroma. Our study demonstrates that via to date unreported effects chloroquine is an effective adjuvant therapy to chemotherapy, offering more efficient tumor elimination and improved cure rates. Chloroquine should be further explored in the clinical setting as its success may help to more rapidly improve the poor prognosis of patients with pancreatic cancer.
INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers worldwide due to the early metastases and strong chemoresistance (1), characteristics that at least in part can be attributed to the presence of a small population of cancer stem cells (CSCs) (2-4). Due to their inherent resistance and/or by maintaining a quiescent state these highly invasive cells evade classic cytotoxic agents (e.g. Gemcitabine) that target mainly differentiated, highly proliferative cancer cells. As a result, a relative enrichment for CSCs during treatment leads to a more aggressive phenotype of the PDAC to which the patient may succumb very soon after or even during the treatment (5-7).

Ex vivo, the population of CSCs can be identified using various cell surface markers, of which the most commonly used are a combination of EPCAM, CD44, and CD24 or the single markers CD133 or ALK4, respectively (3, 6, 8). Moreover, we have previously shown that a subset of the CD133+ CSCs, identified by co-expression of C-X-C chemokine receptor 4 (CXCR4), is driving metastatic spread (6, 9). These invasive CXCR4+ cells respond to CXCL12 (stromal derived factor-1) produced by tumor stroma as well as organs where metastatic lesions form most frequently, e.g. liver (9, 10). In addition sonic hedgehog (SHH) signaling also plays an important role in mediating chemotaxis and regulating epithelial-to-mesenchymal transition, necessary to gain an invasive phenotype (11).

Targeting the regulatory machinery of CSCs should be essential for the development of more efficient clinic-ready therapies. In a screening effort we surprisingly identified the anti-malaria compound chloroquine as an effective treatment modality against pancreatic CSCs. Following a first observational study that reported an inhibitory effect of chloroquine on the pancreatic cancer cell line PaTu II (12), the effects of chloroquine on tumor growth were later confirmed in mouse models of PDAC and attributed to the inhibition of autophagy (13), which is responsible for recycling bioenergetic components to ensure cell survival in nutrient-deprived times or locations (13). For breast cancer and glioblastoma, chloroquine has been suggested to
also have an impact on the cancer stem-like phenotype via inhibition of autophagy (14, 15). However, it has recently been suggested that chloroquine may also function as an inhibitor of CXCR4 signaling (16). Intriguingly, here we demonstrate for the first time in primary human PDAC cells that chloroquine preferentially targets CSCs and that this occurs through a dual, but autophagy-independent mode of action. First chloroquine did not only directly inhibit CXCL12-induced phosphorylation of ERK and STAT3, but also enhanced internalization of the CXCR4 receptor in CSCs. Secondly, we demonstrate chloroquine blocked sonic hedgehog-induced chemotaxis by decreasing Smoothened (SMO) protein levels with subsequent inhibition of EMT in CSCs. These effects translated into remarkable in vivo cure rates in mouse models of human PDAC and virtual abrogation of metastasis.

MATERIALS & METHODS

Primary patient-derived tissue xenografts. Tumor tissues resected from representative patients with PDAC prior to any neoadjuvant radiation or chemotherapy were implanted into immunocompromised mice, to establish low passage tissue xenografts (6, 8). Aqueous solution of Gemcitabine (Lilly, Indianapolis, IN) was administered twice a week (125mg/kg i.p.) for 52 days. Chloroquine (Sigma-Aldrich, St. Louis, MO) was administered as PBS-based solution daily for 21 days (50mg/kg i.p.).

Cells and cell cultures. Above PDAC tissues were also used to obtain primary cells for in vitro studies (6, 8). In addition, established human pancreatic cancer cell lines Panc1, BxPC3, and 8988T were obtained from ATCC, cultured and maintained as previously described (6), and their identity was tested by Short Tandem Repeat Profiling (University of Arizona, Genetics Core Facility, AZ). The selected concentration of aqueous solution of chloroquine (10μM; Sigma Aldrich) was based on the cytotoxicity analysis and administered daily (Fig. S1A).
**In vivo metastasis assay.** Firefly luciferase-expressing PDAC-354 cells were injected into the spleen of immunocompromised mice randomized to control or chloroquine treatment until the end of the experiment. The IVIS Spectrum Imaging System (Caliper Life Sciences, Waltham, MA) was used for weekly *in vivo* luciferase imaging.

**RNA preparation and RT-PCR.** Total RNAs were extracted with TRIzol kit (Life Technologies, Alcobendas, Spain) according to the manufacturer’s instructions and used for cDNA synthesis. Quantitative real-time PCR was performed using primers depicted in Table S1 or as previously published (8).

**Western Blot Analysis.** Total protein extracts were obtained by lysis with Laemmlli buffer and 25-50 μg of protein was separated by SDS/PAGE and transferred to nitrocellulose membranes that were probed with antibodies depicted in Table S2. Cell Surface Protein Isolation Kit (Thermo Fisher Scientific, Waltham, MA) was used according to manufacturer’s instructions to obtain membrane fraction of the protein lysate. Loading amount was determined using an antibody against Na,K-ATPase Antibody (#3010, Cell Signaling Technology, Danvers, MA). Stained gels were scanned and ImageJ was used to quantify the band signals for each sample.

**Flow Cytometry.** Cells derived from human primary cancer cells, or 7-day sphere cultures or from freshly isolated tumors were incubated with primary antibodies (or appropriate isotype-matched control antibodies) listed in Table S2.

**Migration and invasion assay.** Migration and invasion assays were performed as previously published (8).

**Histology.** Tumor samples were paraformaldehyde-fixed, paraffin-embedded, sectioned, and processed for immunohistochemistry. Sections were incubated with primary antibodies, followed by incubation with appropriate secondary antibodies and substrates to enable detection. Nuclear counterstaining was performed using hematoxylin.

**Immunofluorescence.** Primary pancreatic cancer cells were seeded on glass coverslips and cultured overnight with 10 μM/ml chloroquine. Next day, cells were washed with cold PBS.
and fixed with 4% PFA (20min at 4°C). Fixative was removed by washing with cold PBS-TritonX 0.1% and samples were blocked using 5% BSA in PBS-TritonX 0.1%. Incubation with primary antibodies against SMO (ab38686; Abcam) and acetylated tubulin (Sigma-Aldrich) was performed overnight at 4°C. Cells were then washed with cold PBS and incubated with Alexa-Fluor-conjugated secondary antibodies against mouse or rabbit (Life Technologies) at room temperature for 45min in the dark. Samples were mounted in Vectashield containing DAPI and analyzed using an SP5 confocal microscope (Leica, Heidelberg, Germany).

**Plasmids and transfections.** Plasmids used were as follows: LC3II-GFP reporter plasmid (a kind gift of Dr. M. Soengas), Gli-Luc reporter plasmid and GLI1 expression plasmid. Plasmid containing GLI1 encoding cDNA was obtained from I.M.A.G.E. Consortium CloneID 3531657 (17).

**Statistical Analysis.** Results for continuous variables are expressed as mean ± standard deviation (S.D.) unless stated otherwise. Overall comparison of continuous variables was performed with the Kruskal-Wallis test followed by post hoc pairwise comparison using the Mann-Whitney U test. Survival was compared using a Log Rank test. P values < 0.05 were considered statistically significant. All analyses were performed with SPSS 19 (SPSS Inc., Chicago, IL).

**Full description of methods is available as supplementary information.**
RESULTS

Chloroquine eliminates pancreatic cancer stem cells. In an effort to identify more effective treatments against pancreatic CSCs, we established a novel low-throughput drug-screening platform for identifying compounds targeting CSCs (unpublished observations by co-author Irene Miranda-Lorenzo). These analyses demonstrated chloroquine to be one of the promising candidates for depleting CSCs and therefore deserving further in-depth evaluation. CSCs derived from a large panel of primary PDAC tumors were treated with 10μM chloroquine, which significantly decreased expression of pluripotency-related genes such as OCT4, SOX2, and NANOG (Fig. 1A) and markedly reduced their sphere forming ability (Fig. 1B). In addition, the self-renewal ability of chloroquine-treated cells as assessed by the formation of secondary (second-generation) spheres was significantly reduced (Fig. 1C) accompanied by reduced numbers of CSCs as determined by decreased expression of CD133 and ALK4 at mRNA and protein level, respectively (Fig. S2A-D). Consistently, percentages of EPCAM+CD44+CD24+ CSC-enriched cells (Fig. S2E) as well as CSCs identified by autofluorescence as a novel functional biomarker for CSCs (Fig. S2F) were reduced (18). These results demonstrate that chloroquine is capable of significantly decreasing the pool of CSCs in sphere cultures.

To univocally validate the effects of chloroquine on CSC activity we next used the in vivo transplantation assay as the most relevant readout for targeting of CSC. For this purpose increasing numbers of control and chloroquine-pretreated cells (10^3 and 10^4 cells/injection) were transplanted into secondary recipients receiving no further treatment (Fig. 1D & Table 1). Chloroquine-treated cells produced significantly fewer tumors in vivo (Fig. 1E). Decreased in vivo tumorigenicity correlated with reduced numbers of CD133+ cells, which did not revert to control levels during the in vivo incubation period despite no further administered treatment (Fig. 1F). These findings were corroborated by a reduced sphere formation ability of harvested tumors, which became even more pronounced through further passaging (Fig. 1G).
Chloroquine specifically targets pancreatic cancer stem cells. Further analysis of chloroquine-treated cells revealed a decrease in mRNA expression for CyclinD1 and E1 (Fig. 2A) and cell cycle analysis validated that more cells were residing in G0/G1 (Fig. 2B). Comparable numbers of viable cells between control and treated cells (Fig. 2C) suggested that decreased percentage of CSCs was due to reduced proliferation in the presence of chloroquine (Fig. S1B). The anti-proliferative effect of chloroquine was restricted to CSCs as adherent cultures, mostly containing differentiated PDAC cells (8), showed no response to chloroquine on cell proliferation, viability, or EPCAM expression (Fig. 2D-F).

Chloroquine improves outcome in Avatar mice. For further preclinical validation of our findings we used primary PDAC tissue xenografts derived from various patient tissues, also termed Avatar mice were randomized to control, chloroquine alone, Gemcitabine alone, and combined treatment with both compounds (Fig. 3A/B). As predicted by our in vitro experiments, chloroquine alone did not remarkably inhibit tumor growth, which is mainly driven by rapidly proliferating non-CSCs. Only the combination with Gemcitabine demonstrated significant decrease in tumor size. Intriguingly, 50% of the tumors completely disappeared and did not relapse during long-term follow-up. The only exception was the PDAC-253 xenograft, for which all implanted tumors eventually relapsed (Fig. 3B/insert). These data demonstrate that, although ineffective as single agent based on the exclusive targeting of the CSCs, chloroquine represents an effective adjuvant therapy to Gemcitabine in our aggressive preclinical PDAC models.

For more mechanistic studies some tumors were harvested after 2 weeks of treatment and analyzed for CSC content using in vivo and in vitro assays. Chloroquine treatment alone or in combination with Gemcitabine significantly decreased CD133+ CSCs (Fig. 3C) accompanied by decreased expression of ALK4 and down-regulation of the Nodal/Activin signaling pathway (Fig. 3D), we recently showed to be essential for the maintenance of the CSC phenotype (8). Consistently, expression of self-renewal genes was significantly decreased (Fig. 3E). Reduced CSC activity could also be functionally validated for chloroquine alone or in combination with
Gemcitabine by serial in vivo passaging (Table 2). Control or Gemcitabine-treated tumor cells showed a take rate of 100%, while chloroquine-treated cells either alone or in combination with Gemcitabine showed drastically reduced tumorigenicity. These data demonstrate that chloroquine efficiently targets CSCs in vivo.

Chloroquine does not affect autophagy. Previous studies in established PDAC cell lines suggested that chloroquine acts through inhibition of autophagy (13). To verify the relevance of autophagy for targeting CSCs, the same established PDAC cell lines as well as our primary pancreatic CSCs were incubated with chloroquine. Consistent with above studies, chloroquine increased protein levels of autophagy marker LC3IIB in the established cell lines Panc1, 8988T, and BxPC3, but interestingly this was not the case for low-passage primary pancreatic CSCs (Fig. S3A). These data were validated using the LC3II-GFP reporter construct (Fig. S3B/C). Only very high concentrations of chloroquine (100μM) induced LC3II-GFP+ foci, but this concentration was cytotoxic and therefore irrelevant for the observed effects on CSCs (Fig. S1A). Therefore, our data rule out inhibition of autophagy as a driving mechanism for the effects of chloroquine on primary CSCs.

Chloroquine targets the chemokine receptor CXCR4. To further investigate the mechanism of action we next analyzed the role of the CXCL12/CXCR4 signaling pathway (16). First we directly assessed the effect of chloroquine on CXCR4 expressing cells, which showed a more pronounced effect of chloroquine on sphere formation for CXCR4+ cells (Fig. 4A). Consistently, we found significant inhibition of the CXCL12/CXCR4 downstream targets ERK and STAT3 (Fig. 4B/C), which was comparable to the effect achieved with the specific CXCR4 inhibitors ADM3100 (Fig. 4B/D) and AMD3465 (Fig. 4C) and could also be validated in vivo (Fig. 4E). Intriguingly, we did not only observe reduced CXCR4 mRNA expression in chloroquine-treated cells (Fig. S2G), but even more strikingly found a marked reduction in the amount of cell surface displayed CXCR4 in all PDAC cells tested, as well as in CEM, which were used as a positive control as they reportedly express CXCR4 at high levels (Fig. 4F) (19).
Reduced CXCR4 expression on the cell surface was further validated by flow cytometry (Fig. 4G).

CXCL12/CXCR4 signaling plays an important role in metastatic spread of PDAC, as the CXCR4+ epithelial CSCs are activated and stimulated to migrate toward the organs producing high levels of CXCL12 (liver, bone, lungs, etc.) (20). Therefore, we screened primary PDAC cells for their responsiveness towards CXCL12 and other molecules also implicated in the initiation and development of PDAC metastases, such as SHH, TGF-β and Nodal (9, 11). The strongest invasive response was observed for CXCL12 and SHH (Fig. 5A). Chloroquine treatment inhibited CXCL12-induced cell invasion to a comparable degree to AMD3100 (Fig. 5B). Furthermore, chloroquine also inhibited invasion induced by SHH (Fig. 5C), TGF-β (Fig. 5D), and media containing 10%FBS (Fig. 5E), whereas invasion induced by Nodal remained unaffected (Fig. 5F). These effects could be attributed to a significant decline in mRNA levels for EMT markers (Fig. 5G) and again were restricted to CSCs as the migration of adherent cells was unaffected (Fig. S4).

Most importantly, we confirmed our findings in vivo by intraspleenic injection of sphere-derived CSCs constitutively expressing luciferase followed by control (no-treatment) or chloroquine treatment. In vivo bioluminescence imaging performed 6 weeks post intraspleenic injections demonstrated significant luminescence signal detected only in the livers of control mice (Fig. 5H). Consistently, ex vivo analysis of luciferase activity in explanted organs showed significantly less dissemination of luciferase-positive cells in the livers of chloroquine-treated mice (Fig. 5I, left panel), although their tumor burden in the spleen was similar to that seen in control mice (right panel).

Effect of chloroquine on hedgehog pathway. It is important to note that SMO localized in primary cilia activates SHH signaling pathway, while SHH-driven chemotaxis is mediated by SMO protein outside of the primary cilia structure (21). Immunocytochemistry for localizing
SMO in relation to the primary cilia detected by staining for acetylated-α-Tubulin demonstrated the presence of primary cilia in a subset of PDAC cells (Fig. 6A & S5A). Importantly, control cells contained the highest levels of SMO within the primary cilia, while slightly lower levels were found in the cellular membrane and submembranous intracellular compartment outside of the primary cilia. In chloroquine-treated cells SMO was clearly reduced, which was further validated by western blot analysis comparing protein content between the membranous fraction and total cell lysate (Fig. 6B). The reduced levels of SMO in primary cilia suggested that chloroquine inhibits downstream activation of SHH pathway, which we then validated by analysis of the expression of the downstream targets PTCH, GLI1 and GLI2 mRNA, whose levels were significantly decreased by chloroquine (Fig. 6C & S5B-D), whereas inhibition of CXCR4 by AMD3100 did not result in reduced mRNA levels for PTCH, GLI1 and SMO suggesting independent mechanism (Fig. S5E). We also observed decreased PTCH in the membranous fraction following chloroquine treatment (Fig. 6B).

These data were further corroborated using the GLI1-Luc reporter assay demonstrating a significant decrease in the GLI1-promoted-luciferase activity upon chloroquine treatment; an effect that could be rescued by co-transfection of cells with a GLI1-expression plasmid (Fig. 6D). Therefore, although we did not formally prove that chloroquine acts via inhibition of SMO, these data are in line with the hypothesis that chloroquine does not directly target GLI1, but acts upstream of it. Still SHH expression in chloroquine-treated CSCs was significantly higher compared to control CSCs, both at mRNA and protein level (Fig. S5F). Consequently, media derived from 7day-old chloroquine-treated spheres was able to induce expression of PTCH and GLI1 mRNA in tumor-associated stromal cells such as pancreatic stellate cells (PSC), but also in human foreskin fibroblasts (HFF-1) (Fig. S5G). Contrary to CSCs cultures, differentiated PDAC cells demonstrated no major change in the expression levels of SHH or the downstream targets PTCH and GLI1 (Fig. S5H). These data indicate that the overall increase in SHH in response to chloroquine will be modest as the increase is confined to the CSC compartment.
Previous studies demonstrated that metastasis is also promoted indirectly by PDAC cells via paracrine activation of hedgehog signaling in the stromal compartment (22). In return, activated pancreatic stellate cells (PSC) secrete large amounts of Nodal/Activin, which endorses self-renewal and invasiveness of CSCs (8, 23). Based on above data, we hypothesized that chloroquine also targets downstream hedgehog signaling in stromal cells. Indeed, chloroquine reduced SMO and GLI1 protein expression in primary PSCs co-cultured with CSCs (Fig. 6E). Consistent reduction in GLI1 expression could also be observed for tumors treated with chloroquine in vivo (Fig. S6A), which subsequently translated into reduced stroma formation as evidenced by Masson’s trichrome staining (Fig. S6B). Therefore chloroquine represents an important advancement in our armamentarium for tackling aggressive PDAC including its protumorigenic microenvironment.

DISCUSSION

Here we demonstrate that treatment of primary PDAC xenografts with the anti-malarial agent chloroquine preferentially targets the highly aggressive subpopulation of CSCs. Chloroquine inhibited self-renewal of CSCs and essentially abrogated their in vivo tumorigenicity. When administered in vivo using clinically most relevant Avatar mouse models derived from patients with PDAC, chloroquine treatment alone, however, had no major effect on growth of the primary tumor xenografts. This was not surprising considering that the bulk of the tumor represents a heterogeneous population of highly proliferative differentiating and differentiated cancer cells, which we showed do not respond to chloroquine. As the CSC population comprises a relatively small fraction of the tumor, its disappearance would not immediately affect tumor growth or size. Therefore, chloroquine’s ability to eliminate CSCs makes it an ideal adjuvant for Gemcitabine-based therapy that targets the bulk of the tumor and
results in significantly prolonged survival with 65% of the mice being long-term survivors. It should be noted, however, that not all the tested tumors responded equally well to the combined therapy (e.g. PDAC253-derived xenografts). Intriguingly, however, further in vivo studies specifically addressing metastatic activity of CSCs demonstrated that chloroquine as a single agent specifically blocks CSC-driven metastasis, as it prevented their evasion and nesting within the liver despite the fact that growth of primary tumors remained unaffected.

The mechanism through which chloroquine is acting on cancer cells has mainly been attributed to the inhibition of autophagy as a consequence of its lysosomotropic properties (13, 24). Consistently, we also observed a strong inhibitory effect of chloroquine on autophagy in the established PDAC cell lines previously used in the cited studies. However, the same concentration of 10μM had very little to no effect on autophagy in primary PDAC cells. While the reason for the lack of response in primary cells remains unclear, we can exclude that the observed effects of chloroquine in primary PDAC cells are related to inhibition of autophagy. This is in line with recent data in breast cancer cells showing that chloroquine can act independently of its effect on autophagy (25).

In fact, our mechanistic studies demonstrate that the effects of chloroquine at least in part are related to its inhibitory effect on CXCR4/CXCL12 signaling. We found strong inhibition of phosphorylation of ERK as well as inhibition of phosphorylation of STAT3. Even more importantly, we provide for the first time evidence that chloroquine acts directly on CXCR4 by internalization of the receptor, rendering the cells less susceptible to CXCL12 signals. Internalization of CXCR4 receptor is not necessary for the phosphorylation of ERK, but it is necessary for CXCL12-induced chemotaxis (26). Indeed, the observed strong inhibition of CXCL12-induced invasion is in line with our hypothesis that chloroquine prevents the resurfacing of the CXCR4 receptor on the cell membrane or it facilitates its degradation. In addition, decreased levels of CXCR4 mRNA following chloroquine treatment (Fig.S2G) also suggest a lack of feedback mechanism that would enhance the production of CXCR4 in primary cells, or
that it may be blocked by chloroquine. Of course, further validating any of these hypotheses will require investigations in future studies. Besides CXCR4/CXCL12 signaling several other signaling pathways can induce and/or promote metastasis. Hedgehog pathway is necessary not only for maintenance of the stem cell compartment, but also for the induction of metastasis and regulation of EMT (22, 27). Recently published data suggest that inhibition of the CXCR4/CXCL12 signaling pathway may also down-regulate the hedgehog pathway (28).

However, consistent with previous studies (29), we found that AMD3100 treatment did not result in inhibition of the hedgehog pathway in CSCs (Fig. S4E) supporting the notion that the effect of chloroquine could be a direct effect on the hedgehog pathway. Indeed, we were able to show that chloroquine decreased levels of PTCH and SMO protein in the cells surface and the levels of GLI1 protein in the cytoplasm of CSCs. Because the effect on GLI1 protein could be rescued by overexpression of GLI1, we propose that chloroquine negatively regulates SMO protein, subsequently decreasing levels of PTCH and GLI1 proteins, as well as other members of the pathway. Our results indicate that chloroquine acts through internalization of PTCH and SMO protein, respectively, supporting previous studies demonstrating that chloroquine inhibits hedgehog signaling by preventing the segregation of internalized SMO/PTCH complex (30).

Inhibition of downstream hedgehog signaling might in part be responsible for the decreased proliferation of CSCs, as the hedgehog pathway regulates proliferation and cyclinD1 is a direct target of GLI1 (31).

The inhibitory effect of chloroquine on hedgehog signaling was confined to CSCs (Fig. G) and subsequently chloroquine decreased hedgehog ligand-mediated invasive potential of CSCs only, but had no effect on migration of their differentiated progenies (Fig. S5A). Out data support the hypothesis that canonical hedgehog signaling actively promotes metastatic CSCs in PDAC. As metastatic CSC comprise only a small subfraction of pancreatic CSC these data are still conceivable with the observation that bulk PDAC cells in general do not demonstrate an appreciable response to inhibition of canonical hedgehog signaling, as already suggested by
previous studies (32, 33) and our recent study using Smo inhibitors in primary human PDAC xenografts (34). Consistently, our present in vivo studies for the use of chloroquine as single agent also demonstrate little to no effect on the growth of PDAC tumor xenografts, but was already very effective in ablating metastatic activity.

It is important to note that it has been demonstrated that KRAS activation leads to the loss of primary cilium whose presence is necessary for the activation of SMO and canonical hedgehog signaling (35). Correspondingly, Smo is found dispensable for PDAC formation in oncogenic Kras-initiated genetically engineered mouse models (32). However, further studies in mouse models demonstrated that GLI1 expression in PDAC is still of crucial importance as inhibition of GLI1/2 prevented pancreatic intraepithelial neoplasia and PDAC formation (36). Besides the hedgehog pathway, GLI1 expression is driven through a variety of non-canonical mechanism, e.g. oncogenic KRAS, AKT/mTOR/S6K, and TGFβ (36, 37). Our findings indicate that primary cilia remain detectable in a small subset of cells within the CSC population. The presence of primary cilia is consistent with the hypothesis that in this particular subpopulation of metastatic CSCs GLI1 expression is still depending on canonical hedgehog signaling and therefore would be affected by inhibition of SMO. This notion is supported by our data demonstrating no change in GLI1 expression in differentiated PDAC cells lacking primary cilia (Fig. S5G).

Previous studies indicated that cells with active canonical hedgehog signaling are exclusively found within stroma (36), that plays a role in drug delivery by forming a physical barrier (33) and altering chemosensitivity of bulk cancer cells (38), but also appears to provide a supportive niche for CSCs promoting their self-renewal capacity and invasiveness (23). Our study shows that chloroquine was capable of blocking SMO and GLI1 expression in stromal cells derived from patient tissues, as well as in other types of stromal cells. This inhibition was still maintained even in the presence of increased levels of SHH derived from chloroquine-treated CSCs. The molecular mechanism leading to increased SHH expression in PDAC cells following
chloroquine treatment are not entirely clear yet. One interpretation could be that increased SHH levels relate to induced differentiation of CSCs, since SHH is expressed mainly in epithelial committed cancer cells (39). However, others have reported enhanced overall expression of SHH in the CSC compartment, even though this has not yet been directly linked to their stemness phenotype (3).

In summary, elimination of CSCs by chloroquine most likely represents a result of its combined effect on CSCs via inhibition of CXCL12/CXCR4 and hedgehog signaling as well as inhibition of hedgehog signaling in the stroma, which supports CSCs and non-CSCs in a paracrine fashion. Importantly, two phase I/II pharmacodynamic studies are already enrolling patients since early 2013 (NCT01494155 and NCT01777477). Patients with metastatic/unresectable PDAC are receiving a combination of Gemcitabine/Abraxane and hydroxychloroquine during each of the 28-day treatment cycles. Although these studies are focusing on the pharmacodynamics, as most other early phase clinical trials they also use tumor burden as a surrogate endpoint for treatment response. However, targeting the relatively small subpopulations of CSCs may not necessarily produce significant measurable changes in tumor burden, as also seen in our preclinical studies. Still there may be a profound effect on the tumorigenic capacity, but the lack of tumor regression could be misinterpreted as lack of treatment response. Therefore, the addition of anti-CSC agents such as chloroquine to standard chemotherapeutic regimens should be accompanied by the addition of surrogate CSC endpoints.

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REFERENCES


Table 1 – *In vivo* tumorigenicity of control or chloroquine treated cells. *In vitro* pre-treated cells were subsequently injected into secondary recipients using indicated cell numbers. Tumor take rates are provided for the indicated xenografts and respective treatments.

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Table 2 – *In vivo* tumorigenicity in secondary recipients for cells obtained from *in vivo* treated primary PDAC xenografts.

Tumors were harvested at the end of the treatment study and dissociated cells were reinjected into secondary recipients using indicated cell numbers. Tumor take rate and corresponding TIC frequency for the indicated xenografts and respective treatments are provided.

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FIGURE LEGENDS

Figure 1. Chloroquine eliminates pancreatic CSCs. Primary cancer cells were plated in non-adherent conditions with 10μM chloroquine (CQ) or vehicle control (Ctrl). qPCR analysis of expression of OCT4, SOX2, and NANOG (A) and sphere forming capacity (B) after 7 days of treatment. Number of spheres formed in the second generation afterpassaging of the cells derived from control and chloroquine-treated primary spheres without further treatment (C). Primary CSCs derived from 7-day control (Ctrl) or chloroquine (CQ)-treated spheres were assessed for their in vivo tumorigenicity. Tumor take was monitored for 3 months or until tumors had reached 1000mm³ (D). Tumors formed by control- or chloroquine-treated PDAC-354 and 253 cells, (E). Cells obtained from these tumors were analyzed for CD133 expression by flow cytometry (F) and sphere formation ability by serial passaging under non-adherent conditions (G). Asterisk indicates p<0.05; n=3.

Figure 2. Addition of chloroquine preferentially inhibits expansion of CSCs and inhibits in vivo tumorigenicity. qPCR analysis of CyclinsD1 and E1 expression in 7-day spheres (A). Flow cytometry quantification of cell cycle distribution by DAPI (B) and AnnexinV staining of apoptotic cells (C) in spheres and in adherent cultures at day3 (D and E, respectively). Expression of EpCAM in adherent cultures on day 3 as analyzed by flow cytometry (F). Asterisk indicates p<0.01; n=3.

Figure 3. Adjuvant chloroquine improves outcome in mice bearing PDAC patient-derived xenografts. Mice bearing primary xenografts were treated with chloroquine (QC) (black dotted line) and/or Gemcitabine (black continuous line) and measured weekly for changes in size throughout the experiment (A) (data are represented as mean±SEM). Survival curve (B) represents combined data gathered from all tumors except 253 (B/inset). Analysis of cells derived from 2-week treated tumors by flow cytometry for CD133 expression (C) and by qPCR for the
expression of NODAL/ACTIVIN pathway (D) and self-renewal genes (E). Asterisk indicates p<0.01.

**Figure 4. Chloroquine targets CXCL12/CXCR4 signaling pathway.** Analysis of sphere formation capacity of FACS sorted (A/upper panel) CXCR4+ and CXCR4- cells chloroquine (CQ)-treated or untreated (A/graph). WB analysis of pERK/totalERK in cultures treated with CXCL12 for 2-6h (upper panel) and 5min (lower panel) in the presence or absence of chloroquine or CXCR4 inhibitor AMD3100. Quantification of the lower panel WB is shown at the bottom (B). WB analysis of pERK/totalERK in cultures treated with CXCL12 in the presence or absence of chloroquine or CXCR4 inhibitor AMD3465. Quantification of the lower panel WB is shown at the bottom (C). WB analysis of pSTAT3/totalSTAT3 in cultures treated with CXCL12 for 5min in the presence or absence of chloroquine or CXCR4 inhibitor AMD3100 and quantification (D). Immunohistochemistry for pERK in samples derived from tumors treated for two weeks (E). WB analysis of CXCR4 protein expression in the membrane fraction of cell lysate. Na,K-ATPase was using as loading control (F). Quantification of flow cytometry (G/upper panel) analyzed CXCR4 mean intensity (G/graph). Asterisk indicates p<0.01.

**Figure 5. Chloroquine decreases invasive potential of CSCs.** Comparison of invasive capacity of primary PDAC cells chloroquine -pretreated or untreated in the presence of various chemoattractants (CXCL12, SHH, Nodal, TGFb, serum) (A). Primary cells were cultured in the presence of CXCL12 with or without addition of chloroquine or AMD3100, respectively (B). Representative pictures of the wells showing DAPI stained invading cells (left panel) and quantified data (graph). Effect of chloroquine addition to the invasive potential of primary cells when induced by SHH (C), TGFb1 (D), 10%FBS (E) and Nodal (F). qPCR analysis of VIMENTIN, SNAIL and N-CADHERIN expression in day7 spheres (G). In vivo (upper panel) and ex vivo (lower panel) imaging of luciferase activity in the liver (H). Quantification of tumor burden in the liver (the site of metastasis) (left panel) and in the spleen (the original injection site) (right panel) by measuring luciferase activity in organ lysates (I). Asterisk indicates p<0.01.
**Figure 6. Effect of chloroquine on hedgehog pathway.** Confocal analysis of SMO (red) localization and primary cilia (green) by immunocytochemistry in control (A, upper row) and chloroquine-treated cells for 24h (A, bottom row). Scale bar represents 10μm. WB analysis for PTCH and SMO in the membrane fraction of cell protein lysates (B). qPCR analysis of GLI1 expression in spheres at day7 (C). Effect of chloroquine on GLI1 protein was determined by measuring GLI1-Luc activity (D). Effect of chloroquine on expression of SMO and GLI1 in primary PDAC-derived stellate cells was analyzed by WB (left in E) and quantified (right in E). Asterisk indicates p<0.01; n=3.
Figure 1 – Chloroquine eliminates pancreatic cancer stem cells.

A

Fold change in mRNA expression

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B

Number of spheres/mL

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C

Number of 2nd spheres/mL

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D

E

Tumor weight (g)

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F

CD133+ cell content

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G

Number of spheres/mL

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Figure 2 – Chloroquine specifically targets pancreatic cancer stem cells

A

Field change in mRNA expression

B

% of cells in G0/G1 phase

C

% of viable cells

D

% of cells in G0/G1 phase

E

% of Annexin V+ cells

F

% of ECD+ cells
Figure 3 – Chloroquine improves outcome in Avatar mice

A

B

C

D

E
Figure 4 – Chloroquine targets CXCL12/CXCR4 signaling

**A**
FACSorting Strategy
Iso control - APC  CXCR4+  CXCR4-
CXCR4-  CXCR4+

**B**
CXCL12  CXCL12
CQ  CQ
pERK  pERK
Total ERK  Total ERK
2h 2h 4h 4h 6h 6h

**C**
CXCL12  CXCL12
CQ  CQ
AMD3100  AMD3100
pERK  pERK
Total ERK  Total ERK

**D**
pSTAT3  Total STAT3
Ctrl  CQ  AMD3100
185  215  354

**E**
Ctrl  CQ
pERK

**F**
CEM  354  A6L  185
Na+K-ATPase  Na+K-ATPase
CXCR4  CXCR4

**G**
Ctrl  CQ
CXCR4-APC

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Author Manuscript Published OnlineFirst on April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-0948
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5 - Chloroquine targets metastatic activity of cancer stem cells

A) Graph showing the percentage of invading cells in invasion assay. The x-axis represents different concentration of CXCL12 and the y-axis represents the percentage of invading cells. The bars indicate the mean + SEM for three independent experiments.

B) Image and graph showing the fold change in invasion. The x-axis represents different concentration of CXCL12 and the y-axis represents the fold change in invasion. The bars indicate the mean + SEM for three independent experiments.

C) Graph showing the fold change in invasion with SHH (300 ng/ml). The x-axis represents different concentration of CXCL12 and the y-axis represents the fold change in invasion. The bars indicate the mean + SEM for three independent experiments.

D) Graph showing the fold change in invasion with TGFβ (10 ng/ml). The x-axis represents different concentration of CXCL12 and the y-axis represents the fold change in invasion. The bars indicate the mean + SEM for three independent experiments.

E) Graph showing the fold change in invasion with 10% FBS. The x-axis represents different concentration of CXCL12 and the y-axis represents the fold change in invasion. The bars indicate the mean + SEM for three independent experiments.

F) Graph showing the fold change in invasion with Nodal (300 ng/ml). The x-axis represents different concentration of CXCL12 and the y-axis represents the fold change in invasion. The bars indicate the mean + SEM for three independent experiments.

G) Graph showing the fold change in mRNA expression of YWENTIN, SNAI1, and V-CADHERIN. The x-axis represents different concentration of CXCL12 and the y-axis represents the fold change in mRNA expression. The bars indicate the mean + SEM for three independent experiments.

H) Images showing the effect of chloroquine (CQ) on tumor growth in mice. The images on the left are of tumors treated with control (Ctrl) or CQ. The images on the right show the relative light units (RLU) of total protein in liver and spleen.

I) Graph showing the RLU of total protein in liver and spleen. The x-axis represents different concentration of CXCL12 and the y-axis represents the RLU of total protein. The dots represent individual data points, and the bars indicate the mean + SEM for three independent experiments.
Figure 6 - Chloroquine targets hedgehog pathway

A

Control

CQ

B

Ctrl  CQ

PTCH

SMO

Ag staining (loading Control)

C

Fold change in GLI mRNA expression

D

GLI1-Luc normalized activity

E

Primary pancreatic stellate cells co-cultured with CSC-enriched spheres:

215  253  354

Ctrl  CQ  Ctrl  CQ  Ctrl  CQ

GLI1

SMO

betaTUBULIN

Fold change in mRNA expression

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Molecular Cancer Therapeutics

Chloroquine targets pancreatic cancer stem cells via inhibition of CXCR4 and hedgehog signaling

Anamarie Balic, Morten Dræby Sørensen, Sara Maria Trabulo, et al.

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