**Old drugs for new purposes - Chloroquine targets metastatic pancreatic cancer stem cells & their microenvironment**

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Received: July 03, 2014
Published online: September 09, 2014

Pancreatic ductal adenocarcinoma is one of the most aggressive and deadliest carcinomas worldwide. Despite modest response rates and extremely low cure rates, gemcitabine-based therapies still represent standard-of-care. Importantly, chemotherapeutic agents such as gemcitabine target mainly highly proliferative and differentiated cells, while cancer stem cells (CSCs) as the most aggressive and chemoresistant cells in the tumor are regularly spared. CSCs are capable of self-renewing and giving rise to more differentiated progenies, representing the bulk of cancer cells. Thus, CSCs are considered the root of the tumor and the source of disease relapse. To actually achieve long-term progression-free survival, treatment regimens should also target CSCs, either by directly eliminating them or by inducing their differentiation rendering them more susceptible to chemotherapy. Intriguingly, our recent studies revealed that the anti-malaria drug chloroquine preferentially targets CD133⁺ CSCs by depleting their number and self-renewal ability translating into drastically reduced in vivo tumorigenic activity. Even more importantly, chloroquine virtually abolished invasiveness of the metastatic CSC sub-population that co-expresses CD133 and CXCR4. Thus, our data suggest that repurposing chloroquine as a CSC-targeting agent could represent a valuable and imminently available adjuvant therapy for patients with pancreatic ductal adenocarcinoma.

**Keywords:** pancreatic cancer; cancer stem cells; stroma; chemotherapy; resistance; CXCR4; hedgehog

**To cite this article:** Anamaria Balic, et al. Old drugs for new purposes - Chloroquine targets metastatic pancreatic cancer stem cells. Can Cell Microenviron 2014; 1: e227. doi: 10.14800/ccm.227

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**Introduction**

Pancreatic ductal adenocarcinoma (PDAC) is the most aggressive and deadliest cancers worldwide, due to several factors including lack of specific clinical symptoms that would enable on-time diagnosis, rapid dissemination, and strong chemoresistance. The latter has also been attributed to be, at least in part, related to the remarkable desmoplastic response observed in many patients with PDAC [¹]. The overall median survival is less than 1 year from the time of diagnosis[²]. Almost two decades after its initial approval for PDAC, gemcitabine still represents the standard therapy for PDAC and results in a median survival of...
approximately 6 months. Only recently, studies using combination treatments such as folfirinox or gemcitabine/nab-paclitaxel showed increased overall median survival of up to 11 months, but their broad clinical translation is hampered by high costs, current lack of direct comparisons and, most importantly, eventual relapse/progression in most patients (reviewed by [3]).

Finally, serious side effects associated with the cytotoxic regimens also emphasize the necessity for the development of more cancer-specific and effective therapies.

Cancer stem cells (CSCs). In this regard, new hope has recently been spurred by the validation of the cancer stem cell concept for many solid cancers including PDAC.
Specifically, within the cancer cell compartment of PDAC there is a distinct sub-population of cancer cells driving tumor progression and metastases, but also resistance to treatment and/or relapse. This sub-population displays characteristics of stem cells: the ability to self-renew and to give rise to more differentiated progenies actually representing the bulk of the tumor. This led to the introduction of the term cancer stem cells (CSCs) [4], even though it is important to note that these cells do not represent bona fide stem cells nor do they originally arise from stem cells in many instances [4-6]. Instead, these cells are functionally defined by their exclusive long-term in vivo tumorigenic potential [7]. CSCs regularly evade classic cytotoxic agents due to their ability to rapidly eliminate these cytotoxic substances, their exceptional resistance to DNA damage, and their strong anti-apoptotic properties [8]. In addition, by retaining quiescence at least a subpopulation of CSCs efficiently escapes current chemotherapeutics (e.g. gemcitabine) that primarily target highly proliferative cancer cells. Thus, preferential elimination of more differentiated and proliferative cells during such treatment results in the relative enrichment for CSCs within the targeted PDAC tissue, which may eventually translate into a more aggressive phenotype. These observations rationalize the observed rapid relapse after initial tumor regression, to which many patients succumb soon after or even during treatment (Figure 1A) [8-10]. Therefore, only drugs that are also capable of eliminating CSCs (either through direct depletion and/or induction of differentiation into progenies that are more susceptible to cytotoxic agents) may ultimately lead to cure from this aggressive disease. Indeed, novel therapies designed against molecules and/or signaling pathways required specifically for CSC phenotypes may provide new opportunities with little or no damage to the surrounding healthy tissues/organs.

In order to target the CSC population it is prerequisite to be able to identify them within the heterogeneous tumor populations. At present we still lack sophisticated markers or molecules that are uniquely expressed or produced by CSCs, but rather our current marker profiles merely enrich for CSCs. Indeed, we and others have shown that CSCs reside within a sub-population of cells that can be identified using a single (e.g. CD133, ALK4) or a combination of cell surface markers (e.g. EPCAM, CD44, and CD24), respectively [5, 9, 11]. Consistently, high CD133 expression in PDAC correlates with lower 5-year survival rate [12], suggesting that the population of CD133+ CSCs is indeed of crucial functional relevance for tumor biology. Moreover, we have shown that anchorage-independent in vitro culture conditions strongly enrich for pancreatic CSCs as evidenced by an increase in the percentage of the cells expressing the above mentioned cell surface markers and enhanced in vivo tumorigenicity [9]. However, pancreatic CSCs by default constitute a heterogeneous population of cells regarding their aggressiveness and metastatic capabilities, which only adds to the complexity of CSC biology and our inability to comprehensively track, characterize, and eventually target them. Still, we have shown that the highly tumorigenic and gemcitabine-resistant CD133+ CSCs can be sub-divided into two distinct phenotypes: (i) the stationary population that maintains the pool of cancer cells to support local tumor growth and (ii) invasive population of metastatic cancer stem cells expressing CXCR4 that are responsible for the dissemination and development of metastasis [9].

Several new therapeutic approaches against CSCs have also been proposed, but these are frequently hampered by lack of clinical grade compound and/or safety concerns [11, 13]. Intriguingly, however, we have shown very recently that the anti-malarial agent chloroquine can be repurposed as an anti-CSC drug based on its remarkable and preferential ability to eliminate CSCs in PDAC. We found that chloroquine efficiently inhibits self-renewal of CSCs and subsequently depletes in vivo tumorigenicity when transplanted into secondary recipients. The specificity of chloroquine against CSCs is best demonstrated in so-called Avatar mice transplanted with patient-derived xenografts [8]. Treatment of these mice with chloroquine as a single agent significantly reduced the pool of CSCs (i.e. CD133+ cells) after only two weeks of treatment. Consequently, the remaining cells showed little to no ability to self-renew as demonstrated by down-regulation of the Nodal/Actinin signaling pathway [11], reduced sphere formation, and diminished in vivo tumorigenicity. However, it is important to note that the overall tumor mass remained mostly unaffected following chloroquine treatment. Considering that the CSC population encompasses only a small cell population within the tumor, which mainly consists of highly proliferative differentiating and differentiated cancer cells that were not affected by chloroquine, the elimination of CSCs had no significant consequence on the size of the xenograft tumors (Figure 1B). These findings also highlight that the assessment of tumor size is not a suitable readout for CSC-targeting, but those new therapies need to be assessed based on their ability to deplete the CSC content, either directly in the tumor or as circulating CSCs in the blood. On the other hand, metastasis is an important feature of at least a subpopulation of CSCs, which preferentially should also be eliminated by new treatment strategies. Intriguingly, chloroquine alone also blocked CSC-driven metastasis by targeting the highly aggressive subpopulation of CD133+CXCR4+ CSC subpopulation that locates at the invasive front of the tumor from where it drives the metastatic spread [9, 14]. The depletion of the metastatic/invasive activity of CSCs by chloroquine was due to inhibition of the CXCL12/CXCR4 and canonical hedgehog signaling, respectively, both of which have previously been shown to induce and/or promote metastasis.
Specifically, chloroquine treatment lead to the internalization of the CXCR4 receptor resulting in very low levels of CXCR4 surface expression and subsequently a dramatically reduced ability of the cells to respond to the chemoattractant and specific CXCR4 ligand CXCL12. This was demonstrated by their inability to colonize the liver that abundantly expresses CXCL12 and, thus, is the most frequently metastasized organ in patients with PDAC (Figure 1B) [14, 17].

The effects of chloroquine on the Hedgehog pathway are more complex as the canonical cascade of this pathway via Smoothened has been found to be abrogated in PDAC cells due to absence of primary cilia following mutation of KRAS and its subsequent hyperactivation. In contrast, the Hedgehog pathway is highly active in cancer-associated fibroblasts via paracrine signaling from the cancer cells [18-20]. Interestingly, however, we found that a small subset of cells within the CSC population (i.e. the metastatic subpopulation of CSCs) actually maintains primary cilia and, thus, canonical hedgehog signaling. Furthermore, we found that chloroquine treatment induces internalization of the PTCH1 receptor, as well as SMO, thereby diminishing the responsiveness of CSCs to Hedgehog ligands. Consequently, GLI1 levels were diminished in CSCs, which negatively impacted their survival. These findings are in line with previous reports showing that GLI1 regulates cell proliferation and survival of pancreatic cancer cells [18]. Of note, chloroquine also inhibited canonical Hedgehog signaling in the stromal cells resulting in a significant reduction of the stroma burden. Importantly, increasing evidence suggests that disruption of the stroma compartment does not only hamper its supportive role as a CSCs niche, but also removes a physical barrier for drug delivery [21-23]. Thus, our data indicate that besides direct depletion of CSCs, chloroquine might also be inducing CSC differentiation via inhibition of hedgehog signaling and/or elimination of important stemness-promoting signals from the tumor microenvironment (i.e. Nodal/Activin) rendering the subsequently arising progenies more susceptible to chemotherapy.

The ability of chloroquine to eliminate CSCs and the invasive phenotype of PDAC makes it an ideal adjuvant for gemcitabine-based therapies, as the latter only target the more differentiated cancer cells[24]. As illustrated in Figure 1C, Combination treatment not only resulted in the depletion of the CSC population and subsequent inhibition of metastatic potential of the tumor, but also reduced its mass and significantly prolonged survival (more than 65% long-term survivor mice). The combination treatment thus uncovers the full potential of chloroquine making the otherwise incurable PDAC much more susceptible to standard chemotherapies. Therefore, future studies should evaluate the clinical utility of this concept, particularly as chloroquine is a well-studied drug used for many decades and its administration rarely induces serious side effects(e.g. retinopathy and cardiomyopathy; reviewed in [25]), which should be weighed against the poor prognosis of patients with PDAC.

Conflicting interests

The authors have declared that no competing interests exist.

References


