Molecular & Cellular Oncology 3:1, e970097; January 2016; Published with license by Taylor & Francis Group, LLC

Chloroquine anticancer activity is mediated by autophagy-independent effects on the tumor vasculature

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Keywords: anticancer therapy, ATG5, autophagy, chloroquine, clinical trials, melanoma, NOTCH1, vessel normalization

Abbreviations: ATG5, autophagy-related protein 5; CAF, cancer-associated fibroblast; CQ, chloroquine; EC, endothelial cell

Chloroquine is used clinically as an autophagy blocker to potentiate anticancer treatments. However, whether chloroquine acts solely through autophagy-dependent and cancer cell autonomous mechanisms has remained elusive. In a recent study we found that chloroquine reduced intratumoral hypoxia and metastasis, while improving chemotherapy response, largely through an autophagy-independent, NOTCH1-reliant mechanism of tumor vessel normalization.

Recognition of the inherently cytoprotective role of autophagy under conditions of cellular stress[1,2] has led to the contention that blocking cancer cell-intrinsic autophagy may curtail cancer cell resistance to chemotherapy, thereby improving therapy outcome. On the basis of this assumption, which has been supported by an avalanche of *in vitro* and preclinical data,[1,2] first-generation autophagy blockers, e.g., chloroquine (CQ) and its derivative hydroxychloroquine (HCQ), are currently being tested in different clinical trials to potentiate patient response to a variety of anticancer regimens (https://clinicaltrials.gov/). CQ, a well-known antimalarial drug with a demonstrated good safety profile[3] is a weak base that becomes trapped upon protonation in acidic compartments, such as late endosomes and lysosomes. By alkalinizing these acidic compartments, CQ compromises fusion events and disrupts endosomal and autophagic cargo degradation, thus resulting in blockade of the basal and stimulated autophagic flux. The effects of CQ as an autophagy inhibitor are well documented and have been validated by knocking down or knocking out essential autophagy genes, although this comparison has been mainly carried out in *in vitro* studies.[1] In fact, whether the inhibitory effect of CQ on autophagy is the sole or main process entailing its action *in vivo* has remained largely obscure. Moreover, most of the available studies on the theorized anticancer action of CQ have focused on its effects on cancer cells, whereas very little has been reported on the impact of CQ on stromal cells. Considering the emerging appreciation of the relevance of crosstalk between cancer and stromal cells in carcinogenesis and the anticancer therapy response, obtaining insights into the impact of CQ on stromal cells is of particular interest.

To fill these gaps in our knowledge, we evaluated how CQ or genetic inhibition of autophagy affect tumor growth and dissemination and modulate the tumor microenvironment, with particular focus on the interface between tumor cells and the tumor stroma. Using a subcutaneous metastatic melanoma model, which is notoriously known to display an autophagy-addiction for growth and survival,[4] we found that doses of CQ that did not affect primary tumor burden were still capable of reducing the invasiveness and metastatic potential of cancer cells. Further analysis revealed that CQ reduced tumor hypoxia by improving the structural and functional features of the tumor blood vessels through a process known as 'vessel normalization'.[5] The presence of dysfunctional and disorganized tumor vessels is a hallmark of many aggressive cancers and is known to favor the hostile tumor microenvironment characterized by acidosis, hypoxia, and high interstitial fluid pressure, while reducing antitumor immune responses and the efficacy of anticancer treatments.[5] Moreover, the permeable blood vessels facilitate invasiveness and tumor dissemination. In line with this, we found that CQ reduced vessel density and tortuosity and improved endothelial cell (EC) alignment and tight junction formation, while reducing tumor vessel leakiness and increasing tumor vessel perfusion. Importantly, tumor vessel

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Submitted: 09/09/2014; Revised: 09/10/2014; Accepted: 09/10/2014
http://dx.doi.org/10.4161/23723548.2014.970097

normalization in response to CQ also enhanced the delivery and efficacy of chemotherapeutic agents.[4]

To understand whether the effects of CQ on the tumor vasculature were caused by inhibition of autophagy in the cancer cells and/or ECs, we genetically disrupted melanoma cell- or EC-intrinsic autophagy by suppressing the expression of autophagy-related protein 5 (ATG5) in melanoma cells using shRNA or in endothelial cells using EC-specific *Atg5* knockout mice. Compromising autophagy in melanoma cells reduced primary tumor growth and metastasis, but did not normalize the tumor vasculature or prevent cancer cell intravasation. Instead, the metastatic potential of autophagy-compromised melanoma cells was reduced because these cancer cells were unable to survive in the bloodstream. This finding highlighted that blocking cancer cell-autonomous autophagy restrains metastatic dissemination following tumor cell intravasation rather than by preventing it, as is observed after CQ treatment. In stark contrast to the tumor vessel ameliorating effects of CQ, the growth of melanomas with an EC-specific *Atg5* deletion in mice induced a tumor stroma hallmarked by increased angiogenesis and vessel abnormalities. Thus tumor vessel normalization by CQ is a process that is elicited independent of autophagy in the cancer cells or in ECs.

This important finding raised an obvious question; how does CQ affect EC properties in an autophagy-independent fashion? Apart from the inhibition of autophagosome degradation, we found that CQ-treatment induced a dramatic enlargement of the late endosomal/lysosomal compartment in the ECs. Since the endolysosomal compartment is emerging as a dynamic intracellular platform that regulates the signaling properties of key receptors and transcription factors,[6] we investigated whether CQ affected the endosomal localization of a panel of relevant angiogenic modulators in cultured ECs. This analysis revealed that CQ altered endosomal trafficking of NOTCH1 and increased its signaling by eliciting the sustained generation of its transcriptionally active fragment NOTCH intracellular domain 1 (NICD1), leading to a more quiescent EC phenotype. Enhanced NOTCH1 signaling was not phenocopied by targeting autophagy in ECs, further underscoring the autophagy-independent mechanism of NOTCH1 stimulation by CQ. In line with this, both the tumor vasculature normalizing and antimetastatic effects of CQ were completely blunted when melanoma cells were implanted in mice lacking Notch1 in their ECs. This genetic model validated both the essential role of NOTCH1 for the tumor vessel normalization effect of CQ treatment and the importance of this process to prevent metastatic dissemination of the melanoma cells.

By normalizing the abnormal tumor vasculature CQ leads to the generation of a more solid barrier that impedes the entry of cancer cells into the blood circulation, the main transport system for dissemination to other tissues and metastasis. Considering that metastasis is the main cause of death in cancer patients, this newly revealed action of CQ on the tumor vasculature could be of considerable clinical relevance. Additionally, the improved perfusion of the tumor that is achieved by CQ treatment reduces tumor hypoxia, a quintessential property of the aggressive and immunosuppressive tumor microenvironment.[7] Although CQ is endowed with mild immunosuppressive activity[3] the reduction in intratumoural hypoxia may contribute indirectly to improved immune function.[7] In line with this, previous studies have shown the ability of CQ to synergize with certain immunotherapy modalities.[8,9] Further studies are required to validate this novel surmise. Another aspect that requires further attention in future studies is the potential of CQ to curtail the protumorigenic crosstalk between cancer-associated fibroblasts (CAFs) and cancer cells, which is fuelled by heightened autophagy in CAFs.[10]

Finally, we have shown that the ameliorated functionality of the tumor vasculature improves chemodelivery within the

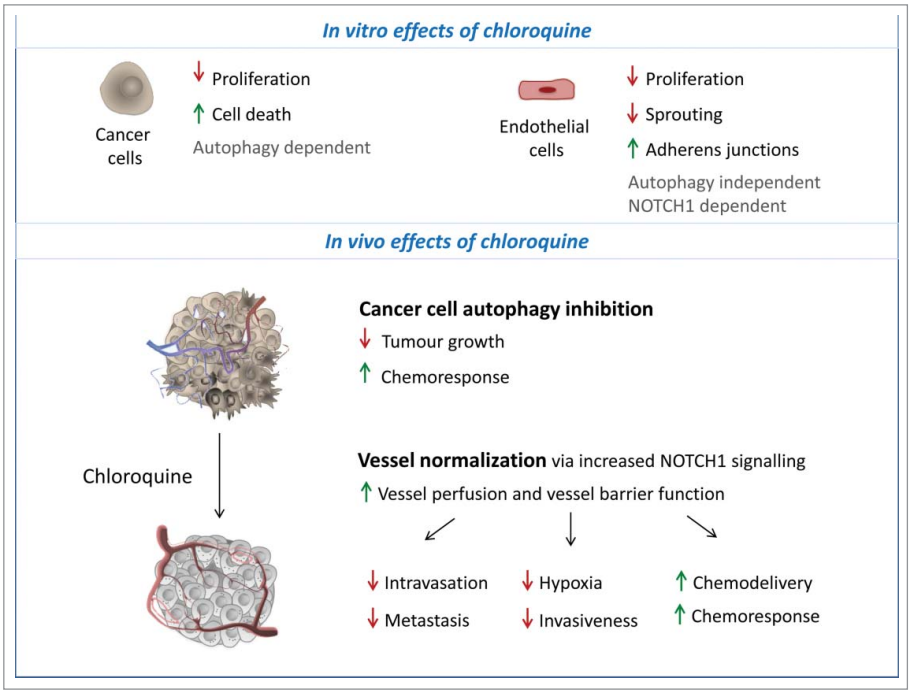

Figure 1. Chloroquine targets the tumor in a dual manner by blocking cancer cell autophagy and normalizing the tumor vasculature. The figure depicts the dual effect of chloroquine (CQ) on the tumor stroma and the underlying mechanisms observed *in vitro* using cultured cells and *in vivo*. In the cancer cells, CQ blocks prosurvival autophagy thereby reducing cancer cell growth. In the endothelial cells (ECs), CQ activates NOTCH1 signaling through autophagy-independent mechanisms resulting in a more quiescent EC phenotype. *In vivo*, CQ exerts a dual effect on the tumor stroma; in addition to blocking cancer cell-intrinsic autophagy, it normalizes the tumor vasculature in a NOTCH1-dependent manner. Through the latter process CQ reduces tumor invasiveness, cancer cell intravasation, metastasis, and hypoxia, and improves chemodelivery and chemoresponse.

tumor and increases the efficacy of chemotherapy.[4] Together with favoring chemotherapy responses, the reduced intratumoral hypoxia could improve oxygen-reliant therapies such as radiotherapy, whose efficacy has been shown to be increased by CQ in preclinical models.[9] Considering that therapeutic strategies aiming at 'normalizing' tumor vessel structure and function rather than destroying the tumor vasculature are attracting increasing attention whereas there are few clinically available agents capable of achieving this effect, the tumor vascular normalizing effects of CQ deserve further validation in clinical settings.

The potential of CQ to act as a autophagy inhibitor in cancer cells and as a vessel normalizing agent (illustrated in **Fig. 1**) advocates for a redesign of the therapeutic schedule and dosage of CQ-based therapies, together with assessment of potential adverse side effects, in order to fully exploit the therapeutic potential of this established antimalarial agent in cancer therapy.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

References

1. Maes H, et al. Autophagy: shaping the tumor microenvironment and therapeutic response. Trends Mol Med 2013; 19(7): 428-46; http://dx.doi.org/10.1016/j.molmed.2013.04.005
2. Amaravadi RK, et al. Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res 2011; 17(4): 654-66; PMID:21325294; http://dx.doi.org/10.1158/1078-0432.CCR-10-2634
3. Lee SJ, Silverman E, Bargman JM. The role of antimalarial agents in the treatment of SLE and lupus nephritis. Nat Rev Nephrol 2011; 7(12): 18-29; PMID:22009248; http://dx.doi.org/10.1038/nrneph.2011.150
4. Maes H, et al. Tumor vessel normalization by chloroquine independent of autophagy. Cancer Cell 2014; 26(2): 190-206; PMID:25117709; http://dx.doi.org/10.1016/j.ccr.2014.06.025
5. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev Drug Discov 2011; 10(6): 417-27; PMID:21629292; http://dx.doi.org/10.1038/nrd3455
6. Dobrowolski R, De Robertis EM, Endocytic control of growth factor signalling: multivesicular bodies as signalling organelles. Nat Rev Mol Cell Biol 2012; 13(1): 53-60.
7. Huang Yet al,, Vascular normalization as an emerging strategy to enhance cancer immunotherapy. Cancer Res 2013; 73(10): 2943-8; PMID:23440426; http://dx.doi.org/10.1158/0008-5472.CAN-12-4354
8. Liang X, et al. Inhibiting systemic autophagy during interleukin 2 immunotherapy promotes long-term tumor regression. Cancer Res 2012. 72(11): 2791-801; PMID:22472122; http://dx.doi.org/10.1158/0008-5472.CAN-12-0320
9. Ratikan JA, Sayre JW, Schaue D, Chloroquine engages the immune system to eradicate irradiated breast tumors in mice. Int J Radiat Oncol Biol Phys 2013; 87(4): 761-8; PMID:24138918; http://dx.doi.org/10.1016/j.ijrobp.2013.07.024
10. Pavlides S, et al. Warburg meets autophagy: cancer-associated fibroblasts accelerate tumor growth and metastasis via oxidative stress, mitophagy, and aerobic glycolysis. Antioxid Redox Signal 2012; 16(11): 1264-84; http://dx.doi.org/10.1089/ars.2011.4243