



Published in final edited form as:

J Invest Dermatol. 2013 September ; 133(9): 2133–2135. doi:10.1038/jid.2013.135.

PUMA: a puzzle piece in chloroquine's antimelanoma activity

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Abstract

Chloroquine can induce cell death in a subset of cancer cell lines, and some melanoma cell lines are quite susceptible. While it is well known that chloroquine impairs lysosomal function and can serve as an autophagy inhibitor, the molecular target of chloroquine and the subsequent cascade of events that leads to cell death are not fully understood. Recent evidence indicates that in melanoma cell lines, chloroquine induces apoptosis by preventing degradation of the pro-apoptotic BH3-only protein PUMA. This finding adds to the unfolding story of chloroquine's mechanism of action as a cancer therapeutic agent.

Although there is clear evidence that chloroquine (CQ) derivatives at micromolar concentrations impair lysosomal function, block autophagy, and elicit cell death in certain cancer cells, the molecular target of CQ derivatives and the molecular mechanism of CQ-associated cell death have not been fully identified. In this issue Lakhter et al. demonstrate a critical role for the pro-apoptotic BH3 only protein p53 upregulated modulator of apoptosis (PUMA) in CQ-associated cell death in melanoma cells (Lakhter *et al.*). This paper provides a provocative step forward in connecting the dots between CQ and apoptosis that may have significant implications for the development of CQ derivatives to treat melanoma and other cancers.

A brief history of chloroquine's use in human disease

CQ is one of the most widely used and successful human drugs in the history of medicine. Since it was first synthesized in 1934 and its implementation as the first effective malaria prophylactic in 1947, it is estimated that hundreds of millions of humans have benefited from CQ and its derivatives (Jensen and Mehlhorn, 2009). Once widespread resistance emerged in malaria strains, CQ, and its better tolerated derivative hydroxychloroquine (HCQ) were repositioned to treat rheumatic diseases (Katz and Russell, 2011). HCQ has been tested in clinical trials as an anticoagulant due to its functional effects on platelet aggregation (Carter and Eban, 1974), as an antiviral against human immunodeficiency virus (HIV) (Paton *et al.*), and as an immunosuppressant for graft-versus-host disease (Khoury *et al.*, 2003). With the exception of malaria and rheumatic disorders, where low doses of HCQ

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provide clinical benefit, randomized trials proved that standard dose HCQ provided no benefit as an anticoagulant, antiviral or immunosuppressive agent, despite strong preclinical data supporting its utility for these indications. Possible explanations for the lack of efficacy in late stage trials include the lack of a dose escalation component to earlier phase studies and lack of mechanism-based pharmacodynamic endpoints that would determine whether the relevant molecular targets had been affected.

More recently, higher doses of CQ derivatives have been tested as anticancer agents. Initial studies in cancer capitalized on the knowledge that CQ derivatives accumulate within and impair lysosomal function, likely blocking multiple cellular processes, including autophagy. Autophagy is a multistep catabolic process that consists of sequestration of damaged organelles and proteins in autophagic vesicles, followed by fusion with lysosomes, leading degradation of autophagic vesicle contents and recycling of sugars, amino acids, and lipids. While it is clear that autophagy has pro-death and pro-survival roles in different contexts, there is increasing evidence that in advanced cancer, autophagy improves the fitness of cancer cells, as it serves to rid the struggling cancer cell of damaged organelles, recycle basic building blocks, and provide an internal source of energy. There is some evidence that basal levels of autophagy are increased in solid tumors(Lazova *et al.*, 2012) as they cope with the metabolic stress of limited resources within the tumor microenvironment and unbridled growth fueled by oncogenes. Certain cancers such as melanoma can have very high levels of autophagy(Lazova *et al.*, 2012). Melanoma may be intrinsically prone to high autophagy levels, since much of the machinery involved in melanogenesis are components of autophagy, rendering most melanocytes professionally autophagic. Cancer therapies induce autophagy, further, providing a rationale for combining anticancer agents with autophagy inhibitors (Amaravadi *et al.*, 2011). Therefore, substantial effort has been devoted to developing strategies to therapeutically target autophagy in cancer cells. However, to date the chloroquine derivatives, CQ, HCQ, and a novel dimeric chloroquine Lys05 (McAfee *et al.*, 2012) are the only pharmacological compounds known to modulate autophagy and elicit cell death in animal models, and therefore they are the only clinically viable autophagy inhibitors. Clinical trials of combinations involving HCQ are underway, and one of the most striking findings has been early signs of unique activity in patients with advanced melanoma compared to other solid tumors when treated with the mTOR inhibitor temsirolimus and HCQ (Amaravadi *et al.*, 2011). This initial activity, and activity demonstrated in numerous preclinical studies, has renewed interest in understanding CQ's mechanism of anti-melanoma activity.

Chloroquine's mechanism of action

At concentrations greater than 10 μ M, numerous extra-lysosomal candidate targets for CQ, which may or may not be related to autophagy, have been reported. These include DNA, glycogen synthase kinase 3 beta (GSK-3 β)(Taha *et al.*, 2008), NADH quinone oxidoreductase 2 (NQO2)(Graves *et al.*, 2002), aldehyde dehydrogenase 1 (ALDH1)(Graves *et al.*, 2002), and chemokine receptor 4 (CXCR4)(Kim *et al.*). Therefore, like most drugs that have utility in medicine, CQ may have multiple molecular targets and therefore multiple mechanisms of action, all of which may be dose-dependent. Human pharmacokinetic studies conducted in patients with rheumatoid arthritis(Munster *et al.*, 2002) and preliminary data

from HCQ trials in cancer patients have demonstrated that low micromolar concentrations of HCQ (<10 μM) are achieved with the highest clinically tolerable doses of HCQ. At these concentrations HCQ may not bind sufficiently to modulate the targets mentioned, but since there is abundant evidence that CQ derivatives accumulate within the lysosomes (McAfee *et al.*, 2012), there is a possibility that autophagy will be blocked to some extent at clinically tolerated doses.

Putting aside the possibility of a dose-dependent molecular target, it is clear that a subset of human cancer cell lines undergo cell death *in vitro* when treated with single agent CQ derivatives. The mechanism of cell death that follows CQ treatment is another “black box”. It is clear that CQ treatment of cancer cells can block autophagy, producing a burst of reactive oxygen species, followed by DNA damage, and activation of DNA damage response-induced apoptosis. Studies have implicated multiple redox-dependent genes as playing roles in the fate of chloroquine treated cells (Bray *et al.*). While these studies have established a potential cascade of events through multiple genes that may play roles in cell death, the molecular underpinnings of CQ-induced cell death are still not clear.

Enhancing PUMA's ability to kill

In this issue Lakhter *et al.* report the effects of high-dose chloroquine (50 μM) on a panel of melanoma and non-melanoma cells. The investigators find that specific melanoma cell lines undergo CQ-induced apoptosis much more readily than normal cells or a sampling of colon, lung or breast cancer cell lines. CQ was found to stabilize PUMA levels post-translationally, and CQ-associated PUMA stabilization requires an intact BH3 domain. Other lysosomal autophagy inhibitors did not stabilize PUMA to the extent that CQ did. Finally, knockdown of PUMA abrogated CQ-associated cytotoxicity implicates PUMA stabilization as at least one important part of CQ's antitumor mechanism of action.

This work raises a number of questions: How is PUMA, a protein typically bound to the mitochondria, modulated by CQ, a compound that accumulates significantly in lysosomes. Could this be a lysosome-independent effect of CQ? Could it be due to binding and inhibition of some of the other targets mentioned above, which are all extralysosomal? While included in the study by Lakhter *et al.*, other inhibitors of lysosomal or autophagic function, including bafilomycin, lysosomal protease inhibitors, and a proximal inhibitor of autophagy, did not stabilize PUMA levels, CQ-induced PUMA stabilization could still be primarily a secondary consequence of lysosomal inhibition. For example, CQ may somehow be more effective than lysosomal deacidification with bafilomycin or lysosomal protease inhibition by blocking autophagic vesicle-lysosome fusion, (somehow) leading to stabilization of PUMA. It is unclear whether other pro-apoptotic BH3-only proteins, such as NOXA, are also stabilized by CQ. Future studies will focus on identifying the protein complexes responsible for BH3 domain-dependent PUMA degradation and whether CQ binds to these proteins. Finally additional studies are needed to determine whether lower concentrations of CQ, such as those achieved in preclinical models and human trials, can also induce a PUMA-dependent cell death.

Nevertheless, this study is important because it shows a clear effect of CQ on PUMA protein degradation, a novel mechanism of action of CQ in human cancer cell lines in which PUMA is not chromosomally deleted and p53-dependent induction of PUMA is intact. If this mechanism is confirmed, measurement of PUMA expression before treatment could identify patients who might be susceptible to CQ-based therapies, and serial measurement of PUMA in tumor tissue could serve as a biomarker of CQ efficacy. Further dissection of the molecular mechanism by which CQ stabilizes PUMA could yield new targets for other therapeutics. As more and more evidence supports the testing of lysosomal autophagy inhibitors in cancer, the puzzling pieces of CQ's mechanism of action should be put together, hopefully avoiding the fate of chloroquine derivatives had in other diseases.

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Clinical Relevance

- Chloroquine (CQ) derivatives have been used to treat a number of diseases, but a dose dependent mechanism of action may explain why they are effective for some disorders and not for others.
- A subset of melanoma cell lines undergo cell death in response to CQ, but the molecular target and mechanism of action of CQ-induced cell death is still not fully understood.
- PUMA-dependent cell death following CQ treatment could provide a rationale for selecting patients for CQ-based therapies based on PUMA expression, and following the effectiveness of novel chloroquine derivatives designed for cancer therapy by measuring PUMA modulation in tumors.