PPT1 Inhibition enhances the anti-tumor activity of anti-PD-1 antibody in melanoma

Gaurav Sharma, …, Dmitry I. Gabrilovich, Ravi K. Amaravadi

*JCI Insight.* 2020. [https://doi.org/10.1172/jci.insight.133225](https://doi.org/10.1172/jci.insight.133225).

Graphical abstract

Find the latest version:

[https://jci.me/133225/pdf](https://jci.me/133225/pdf)
PPT1 inhibition enhances the anti-tumor activity of anti-PD-1 antibody in melanoma

Authors
Gaurav Sharma¹, Rani Ojha¹*, Estela Noguera-Ortega¹*, Vito W. Rebecca¹, John Attanasio², Shujing Liu³, Shengfu Piao¹, Jennifer J. Lee¹, Michael C. Nicastri⁴, Sandra L. Harper⁵, Amruta Ronghe⁵, Vaibhav Jain¹, Jeffrey D. Winkler⁴, David W. Speicher⁵, Jerome Mastio⁶, Phyllis A. Gimotty⁶, Xiaowei Xu³, E. John Wherry², Dmitry I. Gabrilovich⁵, Ravi K. Amaravadi¹#

*These authors contributed equally for second position

Affiliations:
1-Abramson Cancer Center and Department of Medicine; University of Pennsylvania, Philadelphia, PA,
2-Department of Systems Pharmacology and Institute of Immunology, University of Pennsylvania
3-Department of Pathology; University of Pennsylvania,
4-Department of Chemistry, University of Pennsylvania
5-Wistar Institute, Philadelphia, PA.
6-Department of Biostatistics, Epidemiology & Informatics, University of Pennsylvania

#Corresponding author:
Ravi K. Amaravadi, MD
852 BRB 2/3
421 Curie Blvd
Philadelphia, PA 19104
Ravi.amaravadi@pennmedicine.upenn.edu
(215)796-5159

Key words: PPT1, macrophage polarization, chloroquine, melanoma, anti-PD-1 ab.
**ABSTRACT:** New strategies are needed to enhance the efficacy of anti-programmed cell death protein (PD-1) antibody (Ab) in cancer. Here, we report that inhibiting palmitoyl-protein thioesterase 1 (PPT1), a target of CQ derivatives like hydroxychloroquine (HCQ), enhances the antitumor efficacy of anti-PD-1 Ab in melanoma. The combination resulted in tumor growth impairment and improved survival in mouse models. Genetic suppression of core autophagy genes, but not Ppt1, in cancer cells reduced priming and cytotoxic capacity of primed T cells. Exposure of antigen primed T cells to macrophage conditioned medium derived from macrophages treated with PPT1 inhibitors enhanced melanoma specific killing. Genetic or chemical Ppt1 inhibition resulted an M2 to M1 phenotype switching in macrophages. The combination was associated with a reduction in myeloid-derived suppressor cells (MDSCs) in the tumor. Ppt1 inhibition by HCQ, or DC661, induced cyclic GMP-AMP synthase (cGAS), stimulator of interferon genes (STING), tank-binding kinase 1 (TBK1) pathway activation and the secretion of interferon β (IFN-β) in macrophages which was a key component for augmented T cell-mediated cytotoxicity. Genetic Ppt1 inhibition produced similar findings. These data provide the rationale for this combination in melanoma clinical trial and further investigation in other cancers.
Introduction

While there have been extensive efforts to combine other T cell stimulating factors with anti-PD-1 Ab, there is an increasing interest in identifying T cell-independent strategies that will augment the efficacy of anti-PD-1 Ab. Tumor cell autophagy has been identified as a major resistance mechanism to targeted therapy and chemotherapy. CQ and HCQ are the only drugs that have been tested as autophagy inhibitors in clinical trials in cancer patients, however there are conflicting reports about whether as a single agent CQ derivatives augment, impair, or have no effects on anti-tumor immunity (1, 2).

Recently, we identified the major molecular target of CQ derivatives as the lysosomal protein PPT1 (3, 4). Meanwhile, multiple clinical trials involving HCQ in combination with chemotherapy or targeted therapy have demonstrated the safety of HCQ combination regimens and some preliminary anti-tumor activity in patients (5, 6). To date, the role of PPT1 in cancer immunotherapy has remained unexplored. Therefore, we conducted preclinical studies with the clinically used PPT1 inhibitor HCQ in two different immunocompetent mouse melanoma models and demonstrated an enhancement of tumor response when anti-PD-1 Ab was combined with HCQ. In vitro, HCQ, a more potent PPT1 inhibitor D661 or siPpt1 were all able to convert M2 to M1 TAMs, and conditioned media from macrophages treated with Ppt1 inhibitors were able to enhance T cell mediated cancer cell killing. Interestingly, the combination of HCQ and anti-PD-1 Ab was resulted in a change in TAM polarization and a significant reduction in MDSCs infiltration in vivo while each single agent did not. The mechanism by which PPT1 inhibitors produced macrophage phenotype switching was dependent on mitochondrial calcium release and p38 activation. PPT1 inhibition also induced the cGAS, STING, TBK1 pathway to induce IFN-β release from macrophages. Ppt1 inhibition-induced IFN-β release was critical for the augmentation of antigen primed T cell killing of melanoma cells. These data suggest that this combination which
can be immediately tested in the clinic could provide an alternative rational combination approach for melanoma immunotherapy.

**Results**

In order to determine if autophagy inhibition could augment the efficacy of immunotherapy, we treated B16 melanoma tumors with IgG, HCQ, anti-PD-1 Ab and anti-PD-1 Ab and HCQ. Only the combination of anti-PD-1 Ab and HCQ significantly impaired tumor growth (Figure 1A) without any sign of toxicity (Supplemental Figure S1A). Tumors harvested at the end of the experiment were significantly smaller with combination compared to monotherapy arms (Figure 1B and 1C).

Unlike a previous report (7), we found no change in mTOR signaling or autophagy induction in B16 melanoma cells treated with anti-PD-1 Ab (data not shown), and anti-PD-1 Ab + HCQ did not show any cytotoxicity in B16 melanoma cells in vitro (Supplemental Figure S1B). In B16 tumors, however, the LC3II/I ratio was increased and p62 level was reduced albeit to a modest degree with anti-PD-1 Ab reflecting the induction of autophagy *in vivo* (Figure 1D). Accumulation of autophagic vesicles was observed in electron micrographs of a tumor biopsy of a melanoma patient treated with pembrolizumab (Supplemental Figure S1C). Anti-PD-1 Ab and HCQ produced significantly increased apoptosis in tumor tissue (Figure 1D) and increased the survival of mice compared to anti-PD-1 Ab alone (Figure 1E).

To understand the effects of autophagy inhibition on T cell priming or effector T cell function, we performed an in vitro priming and co-culture experiment using either C57BL6J splenocytes, or purified CD8+ T cells isolated from spleens. For priming we exposed splenocytes or CD8+ T cells to irradiated B16 cells. Next these primed splenocytes or CD8+ T cells were cultured with live B16 cells and cytotoxicity was measured (Figure 2A). Antigen-primed splenocytes or non-specifically (concanavalin A) activated splenocytes were co-cultured in the absence or presence of HCQ with live B16 cells. The addition of HCQ after priming did not impair T cell mediated killing of tumor cells (Figure 2B). Neither HCQ, the dimeric chloroquine DC661
(3), nor anti-PD-1 Ab administered before or after priming
either augmented or blunted antigen
specific T cell killing of B16 cells (Figure 2C). To study the effects of autophagy inhibition in this
system we systematically knocked down key autophagy genes involved in each of the major
autophagy protein complexes that coordinately assemble the autophagosome, including *Ulk1*,
*Pik3c3* (*Vps34*), and *Atg7*. Knockdown of these three genes in B16 cells produced reduced
autophagic flux as evidenced by increase in p62. While Lc3b-II levels did not change much with
si*Ulk1* or si*Pik3c3* as is often observed (8, 9), there was significant loss of Lc3b-II expression as
expected with si*Atg7* (Figure 2D). Splenocytes or T cells primed with B16 cells with any of the
three autophagy genes knocked down produced significantly less interferon gamma upon
stimulation than B16 cells exposed to siNon-target control (Figure 2E). In contrast, knockdown of
*Ppt1*, the lysosomal target of chloroquine derivatives, in B16 cells resulted in no significant
difference in T cell priming, as evidenced by similar interferon gamma secretion compared to
siNon-target knockdown (Figure 2F,G). Knockdown of *Ulk1*, *Pik3c3*, or *Atg7* in B16 cells during
priming resulted in significantly reduced T cell mediated killing of proliferating B16 cells expressing
the same siRNAs. In contrast knockdown of *Ppt1* did not impair antigen-primed T cells from killing
B16 si*Ppt1* cells (Figure 2H). Next, to determine if autophagy induction in tumor cells by anti-
PD-1 Ab is a major contributor to the reduced efficacy of anti-PD-1 therapy, CRISPR- cas9 gene
editing was used to knock out (KO) an essential autophagy gene *Atg7* in B16 cells (Figure 2I). As
was found to be the case with si*Atg7*, B16 Atg7 KO cells showed a reduced ability to prime
syngeneic splenocytes compared to B16 WT cells (Figure 2J). Tumors were generated in the
flanks of C57Bl6/J mice using B16 Cas9 wild type (WT) and B16 KO *Atg7* mouse cell lines. While
treatment with anti-PD-1 + HCQ significantly suppressed tumor growth rates compared to
monotherapy in B16 *Atg7* WT cells, no such augmentation of anti-PD-1 efficacy was observed in
B16 *Atg7* KO tumors treated with anti-PD-1 (Figure 2K). Immunophenotyping of the tumors
described above demonstrated that anti-PD-1 Ab significantly increased the percentage of CD8+
T cells in both B16 *Atg7* WT tumors and B16 *Atg7* KO tumors (Figure 2L). There was no significant
difference in the % CD8+ T cells in B16 Atg7 WT tumors from mice treated with anti-PD-1 Ab and HCQ compared to anti-PD-1 Ab alone. Whereas in the spleen there was increased CD8+ T cells with all treatments given, compared to control in both Atg7 WT and Atg7 KO tumors (Figure 2L). Similarly, there were no significant differences in Ki67+ or granzyme B positive CD8+ T cells in either the tumors or the spleens across all treatments (Supplemental Figure S2). Taken together these findings suggested that the enhanced efficacy of HCQ and anti-PD-1 Ab was not due to a direct effect on T cells nor through autophagy inhibition in tumor cells.

A recent study suggests that single agent CQ modulates the phenotype of tumor associated macrophages from a pro-tumorigenic (M2) to an anti-tumorigenic (M1) phenotype or mouse macrophages in vitro and in vivo (1). To test this, Raw 264.7 mouse macrophages were polarized to an M2 phenotype and treated with HCQ or DC661. Both HCQ and DC661 resulted in M2 to M1 phenotype switching as evidenced by significance changes in inducible nitric oxide synthase (iNOS), Arginase 1 (ARG1) and RETNLA/FIZZ1 (Fig. 3A). Morphologically, there was a striking change in M2 polarized macrophages treated with HCQ or DC661, with cells taking on a spindle shape with increased pseudopodia resembling M1 polarize cells (Figure 3B). These findings were reproduced in mouse bone marrow-derived macrophages (BMDMs) (Figure 3C). Ppt1 knockdown also produced a 25-fold increase in iNOS and 2-3-fold decrease in RETNLA/FIZZ1, reflecting effective change in polarization from an M2 to M1 phenotype (Figure 3D). Knockdown of core autophagy genes Ulk1, Pik3c3 and Atg7 also produced changes in M2 to M1 macrophage polarization (Figure 3E). Ppt1 KD produced 4-6 times the change in iNOS expression compared to knockdown of other core autophagy genes suggesting a more efficient macrophage polarization switch was achieved by targeting Ppt1.

CD8+ T cells play a major role in the elimination of tumor cells and regression of tumors by their cytotoxic activity (10). To determine if drug induced changes in macrophage phenotype could contribute in the elimination of tumor cell by T cells, splenocytes were co-cultured with
irradiated B16 cells and after priming, co-cultured with live B16 cells in the presence or absence of macrophage conditioned media (MCM), collected from control, HCQ or DC661 treated macrophages (Figure 3F). The exposure of HCQ or DC661 treated MCM to antigen-primed splenocytes significantly enhanced antigen-primed splenocyte mediated killing of B16 cells compared to antigen primed splenocytes exposed to vehicle-treated MCM (Figure 3F, Supplemental Figure S3). Unlike a previous report which showed CQ can change TAM M2 to M1 phenotype in vivo (1), but in concordance with another published report (2) which showed CQ derivatives had no effects on immune infiltrates in the tumor microenvironment, we found no change in immune infiltrates with HCQ or anti-PD-1 alone. However, the combination of anti-PD-1 and HCQ produced a significant 3-fold increase in M1/M2 ratio. The combination also significantly reduced PMN-MDSCs (Figure 3G), which play a major immunosuppressive role in the tumor microenvironment (11). There were no significant changes in tumor monocytic (M)-MDSC, eosinophils, dendritic cells (DCs), CD4+ T cells, or CD8+ T cells, (Supplemental Figure S4A-B). However, the percentage of NK cells increased significantly with anti-PD-1 Ab and HCQ as compared to IgG control or anti-PD-1 Ab alone (Supplemental Figure S4B-C). Label free proteomics of whole B16 tumors demonstrated that proteins related to macrophage biology were significantly increased or decreased with combined anti-PD-1 Ab + HCQ treatment compared (Supplemental Figure S4D). These included a 4 fold increase in Rab6a, required for TNF secretion in M1 macrophages (12), a 2 fold increase in Golga7, essential for palmitoylation of proteins (13); a 2-fold decrease in matrix metalloprotease 12 (MMP12) that stimulates MDSC expansion (14), and a 4-fold decrease in leukemia inhibitory factor (LIF), that supports immunosuppressive TAMs (15).

We next used the B\textit{Raf} \textsuperscript{CA}, P\textit{ten}\textsuperscript{loxP/lox}, \textit{Tyr::CreER}\textsuperscript{T2} mouse melanoma model. In this model painting the skin of mice with 4 hydroxytamoxifen (4-HT) activates Cre recombinase, and melanocytes express mutant \textit{Braf} and lose \textit{Pten}. Spontaneous melanoma tumors arise on the
skin of these mice with a 100% penetrance. In concordance with B16 tumor model we found that HCQ significantly enhanced the anti-tumor response of anti-PD-1 Ab (Figure 4A-B), with no sign of toxicity (data not shown). A significant reduction of Ly6G/Ly6C+ MDSCs was also observed with the combination using immunohistochemistry staining of excised tumor tissue (Figure 4C-D).

Interestingly, HCQ with anti-PD-1 Ab reduced the infiltration of M-MDSCs (CD45+CD11b+Ly6G\textsuperscript{low}Ly6C\textsuperscript{high}) (Figure 4E). There was a significant reduction in tumor CD45+CD11b+ Ly6C+Ly6G-CX3CR1+ monocytes and spleen eosinophils (CD45+CD11b+siglec F+) and no significant change in DCs (CD45+CD11b+CD11c+) and PMN-MDSCs (CD45+CD11b+Ly6C\textsuperscript{low}Ly6C\textsuperscript{high}) (Supplemental Figure S5A-B). There was no significant change in CD8+Ki67+, CD8+Granzyme+, CD4+ and Foxp3+ (Treg) T cells compartment in the spleen and tumor (Supplemental Figure S6). Taken together these data suggest that CQ derivatives that target PPT1 enhance the anti-tumor efficacy of anti-PD-1 Ab by targeting both the tumor cell and multiple immunosuppressive myeloid subsets. These effects result in enhancement of T cell mediated killing without major changes in the T cell populations. Taken together these results show that PPT1 inhibition changes macrophage from an M2 to an M1 polarization state and significant reduces tumor MDSC infiltration in vivo. It also raises the possibility that PPT1 inhibition stimulates macrophages to secrete a factor that activates enhances T cell cytotoxicity.

Having established a link between PPT1 inhibition in myeloid cells and T cell killing, we next turned our attention to the mechanism by which chloroquine derivatives were augmenting anti-tumor immunity. Previous work showed that chloroquine activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) signaling in M2 macrophages through a lysosomal calcium channel transient receptor potential cation channel mucolipin 1 (TRMPL1). This report suggested that lysosomal inhibition induces TRPML1-dependent calcium release, which leads to phosphorylation of p38, and phosphorylation of p-NF-κB p65 that in turn regulates the transcriptional profile of macrophages that manifests in the M2 to M1 phenotype switch. In M2
polarized macrophages, HCQ or DC661 produced significant intracellular calcium release (Figure 5A). HCQ, DC661 or the weak PPT1 inhibitor hexadecylsulfonyl fluoride (HDSF) treatment resulted in increased levels of phospho-p38 (Figure 5B). In addition, knockdown of Ppt1 also resulted in increased levels of phospho-p38 compared to siNon-target control (Figure 5C). PPT1 inhibition with HCQ or DC661 induced phosphorylation of p-NF-κB p65, a gatekeeper transcription factor for the pro-inflammatory phenotype (Figure 5D). Inhibition of p38 completely abrogated the M2 to M1 phenotype switch elicited by treatment with HCQ or DC661 (Figure 5E).

To understand how PPT1 inhibition was inducing phosphorylation of p38, we first determined if calcium release following Ppt1 inhibition was required for phosphorylation of p38. Co-treatment of M2 polarized macrophages with Ppt1 inhibitors and BAPTA, a calcium chelator (Figure 5F), or W7 a calmodulin inhibitor (Figure 5G) abrogated HCQ or DC661-induced phosphorylation of p38. However contradicting previous report (1) we found that verapamil, a TRMPL1 calcium channel blocker was not able to abrogate HCQ- or DC661-induced phosphorylation of p38 (Figure 5H). As a positive control, the TRPML1 agonist MK6-83 also induced phosphorylation of p38, which was completely abrogated by TRMPL1 inhibitor verapamil. Upstream of TRMPL1, is the lipid kinase PIKfyve which phosphorylates Phosphotidylinositol (PtdIns)-(3)P to PtdIns(3, 5)P2. TRPML1 activity requires binding to PI(3,5)P2, the lipid product of PIKfyve (16). PIKfyve inhibition with vacuolin-1 was not able to blunt HCQ or DC661-induced or MK6-83 induced phosphorylation of p38 (Figure 5I). Since these experiments determined that TRMPL1 was not likely responsible for the calcium release in macrophages treated with Ppt1 inhibition, we next investigated other subcellular compartments that could be responsible for the calcium accumulation found with Ppt1 inhibition. The ER calcium channel blocker ryanodine was unable to prevent DC661-induced activation of phospho-P38. However, CGP37157, an inhibitor of the mitochondrial Na⁺–Ca²⁺ exchanger (NCLX), completely abrogated the increased p38 phosphorylation induced by lysosomal inhibitors (Figure 5J). These results support lysosomal
inhibition is associated with calcium release from the mitochondria, activation of p38 which is required for macrophage phenotype switching.

For an unbiased approach to identify how macrophage activation following Ppt1 inhibition could lead to augmented T cell killing, we performed secretome analysis on M2 polarized macrophages grown in 0.01% serum. First, we confirmed under these conditions that conditioned medium from macrophages treated with HCQ or DC661 was still able to augment antigen-primed T cell killing (data not shown). Macrophage conditioned media was subjected to proteome analysis. Comparison of HCQ versus control showed 64 proteins significantly changed proteins (fold change > 2 and p-value < 0.05), while a comparison of DC661 verses control showed 47 significantly changed proteins out of a total of 3,166 proteins identified with high confidence (Figure 6A).

We focused our attention on IFN-β, since it has been previously linked to T cell function (17). PPT1 inhibition by HCQ or DC661 treated M2 macrophages produced a significant increase in secreted interferon β secretion compared to vehicle control (Fig. 6B). IFN-β production can be increased with the activation of the cGAS/STING/TBK-1 pathway (18). We observed that the Ppt1 inhibitors HCQ and DC661 treatment induced significant increase of cGAS in 8 and 24 hours. Unlike DMXAA, a known STING ligand which leads to degradation of STING at 24h, HCQ or DC661 treatment instead resulted in sustained expression of STING protein. Phosphorylation of TBK1 was detected transiently at 8 hours following HCQ treatment, and more persistently at 8 and 24 hours following DC661 treatment (Fig. 6C). Ppt1 knock down mimicked HCQ and DC661 treatment and showed similar increase in cGAS and p-TBK1 induction and maintained STING level (Fig. 6D). The role of PPT1 inhibition by HCQ and DC661 induced c-GAS/STING/TBK-1 upregulation and subsequent IFN-β production was further supported when co-treatment with STING inhibitor C-176 or TBK-1 inhibitor GSK-8612 resulted in abrogation of IFN-β production by HCQ and DC661 or DMXAA (Fig. 6E). Next, we optimized an anti–IFN β neutralization
antibody. We treated mouse splenocytes with exogenous interferon β in the absence and presence of neutralizing antibody and measured phospho-STAT1 as a readout of interferon β activity (Fig. 6F). Finally, we repeated the primed T cell-tumor cell co-culture experiment as described in Fig. 3E. Antigen-primed T cells exposed to conditioned medium from M2 polarized macrophages treated with HCQ, or DC661 exhibited significantly better tumor cell killing than control. This augmentation of cell killing was completely abrogated when anti-IFN-β antibody was co-administered with the conditioned medium (Fig. 6G).

Discussion

Our findings show that PPT1 inhibition likely augments tumor immunity by at least three different means: by causing an M2 to M1 macrophage polarization switch, by reducing the number of MDSCs in the tumor microenvironment, and by inducing IFN-β release from macrophages that stimulates T cell mediated killing (Figure 6H). We also found a significant increase in the infiltration of NK cells into the tumor microenvironment which could also be directly related to IFN-β production induced by chloroquine derivatives. There is a large body of literature that demonstrates that macrophages produced type I interferons such as IFN-β (17), and that type I interferons directly activate CD8+ cytolytic T cells (19-21). These studies and the results of our studies demonstrate that macrophages secrete 1-30 pg/mL of IFN-β, upon stimulation, but this is enough to significantly augment cytotoxic activity of antigen primed T cells. In the combination of anti-PD-1 Ab and PPT1 inhibitor, the anti-PD-1 Ab is likely reinvigorating the T cells and the PPT1 inhibition is further augmenting the efficacy of these antigen primed T cells through macrophage dependent type I interferon.

Unlike Chen et al. we did not see single agent anti-tumor activity with HCQ in two different mouse models of melanoma. This is in line with the lack of evidence of single activity of HCQ in the clinical melanoma. Although numerous reports have shown that lysosomol inhibition or deletion of autophagy genes can impair tumor growth in MAPK mutant cancers (22) as single
agents, in some contexts, deletion of autophagy genes such as Atg7 is well tolerated by tumor cells, and in most models HCQ does not produce single agent antitumor activity (23). Previous findings suggest that Atg7 depletion leads to enhanced major histocompatibility complex class I (MHC I) presentation in tumor cells, but reduced MHC II expression (23). A recent paper showed that in pancreatic cancer autophagy inhibition can upregulate MHC class I and recruit T cells into the tumor microenvironment. In this pancreatic cancer study, autophagy inhibition with chloroquine or genetic autophagy inhibition was unable to augment anti PD-1 antibody antitumor activity, but significantly augmented the activity of combined anti PD-1 Ab and anti-CTLA-4 antibody (24). In our melanoma study we see clear augmentation of T cell priming with PPT1 inhibition but not with genetic inhibition of upstream autophagy genes. B16 expresses low levels of MHC I and MHC II and the studies here suggest that priming of CD8+ T cells by irradiated B16 may require MHC class II expression (25). It is important to note that although the in vivo findings in B16 tumors are only with Atg7 KO conditions, but given the lack of efficacy in T cell priming and antigen primed T cell mediated cytotoxicity when any of the core autophagy genes Ulk1, Vps34, or Atg7 were knocked down in vitro it is also unlikely that PD-1 treatment would be more effective in tumors deficient in these autophagy genes. Since Ulk1, vps34, and Atg7 represent the key nodes of the autophagy pathway, these results indicate that the effects of PPT1 inhibitors are independent of the autophagy pathway in tumor cells at least in the B16 model.

Here we chose B16 and the Braf<sup>Cav600e</sup>Pten<sup>lox/lox</sup> GEMM model as examples of immune cold tumors that are poorly responsive to anti-PD-1 Ab. This is a group of tumors that is sorely in need for new therapies and therefore are relevant models for our purpose. While the combination of anti-PD-1 and PPT1 inhibition by HCQ did not result in significant change in the number of activated T cells as assessed by immunophenotyping, the clear changes in macrophage populations could also have a significant impact on anti-tumor activity. Our finding that PPT1 inhibition leads to
calcium release warrants further study, which we have started in our laboratory. It is known that TRPML1 is a palmitoylated protein, and in humans mutation or loss of this protein leads to a lysosomal storage disorder with a similar phenotype as mutations or loss of Ppt1. Therefore, it may be that PPT1 directly regulates TRPML1. Our study shows that TRPML1-independent calcium release and activation of phospho-p38 is required for PPT1 inhibitors-mediated secretion of IFN-β by macrophages. This calcium release may be critical also for the susceptibility of MDSCs to lysosomal inhibition. MDSCs express high levels of calcium-dependent proteins such as S100A8/A9. Further work is needed to understand how PPT1 inhibitor induced calcium release regulates MDSC viability. It has been previously reported that activation of cGAS/STING/TBK-1 pathway leads to the degradation of the STING protein via the lysosome (26). Therefore, PPT1 inhibition with HCQ and DC661 in our study with no observed degradation of STING protein strongly supports that sustained cGAS→STING→TBK-1 signaling and IFN-β production is the mechanism by which these agents enhanced both CD8+ T cell activity and the efficacy of anti-PD-1 in these models. Given the excellent tolerability of HCQ combined with either targeted (5) or chemotherapy regimens (6), there is now sufficient rationale to launch clinical trials of combined CQ derivatives and anti-PD-1 Ab. Our group has launched the LIMIT melanoma trial (NCT04464759).

Material and Methods

Reagents

DC661 was provided by the laboratory of Jeffrey Winkler. Purity of sample was determined by
NMR spectroscopy and LC/MS. Mouse melanoma B16-F10 (CRL-6475) and Raw 264.7 (TIB-71) were purchased from ATCC. Cell lines were tested for *Mycoplasma* biannually and authenticated using short-tandem repeat fingerprinting. *Atg7* KO B16 cells were generated using Crispr/cas9 editing. *PpT1* siRNAs pool#1 (sigma EMU085041)

CAGCATCTTCTTGGCAGACATAAAATCAAGAGAGGTTGCTGTAATGAGTCTCAACAAGAAGAAC
CTGATGACCCTCAAGAGTGGATGATGGAAATTCTTTATGATTCCATTGTGGACCCTGT
CGACTCTGAGTGTTTGGGATTTCAGAAGTGCCCGATATTCTATGATTCCCTCTCCCTCCAG
GAGAGCAGACTCTTACACAGAGGACCCTGGGCTAAAGAAAAATGGAACAAAGCAGGAAAG
CTAGTTTCTGGAAGAAGGGACCCTTCAATATCTAAAGATGTTTATGCTGCC
ACATCATACCTTTCTTAATGTGACTGCCCCTGGGACTTTATAGCAGAGTTCATGAAACCACGC
TCTCCAAGCCATGTACTAGTTCATGCTCAGGCTGACTCTAATCTAGCCTGCAACCAGC
CCTTCTCTCCTCTTTATCATACTACATACCTCTACTTGGAAGATCTAAGATCTCAATCTTATCAC
TTGCCGCTCT. *PpT1* siRNA pool#2 (santacruz sc-142398)

AGGCCGGAAAGGATAAGATGGAGATCTTAGATCTTTCCAAAGTAGGGGTAGTGGTAGATGATAA
GAGGAGAGAAAGGCTGGTGCAGCTAGATTAGAGTTCAGGCTGAGCATGAACTATGTAC
ATGGCCTTGAAGAGCCTGTTTCTGACTGAACTCTGCTATAAAGTGCCAGGGCATCCTTAAG
AAAAGGTAGATGATGTTGGGAGCTAAACCATTCTTTAGATATTTGAAGATGGTCCCCTTCTTAG
CCAGAAACACTAGCTTTTCTGCTTTTGTCCATTATTTCTTTAGCCCCAGGGGGTCCTCTGTAT
AGAGTGCTCTCCCTGGAGGGGAATGGTTCTTCTTACTGTTGCCACTTCTGTTAGAAATCCAAACC
ACTCAGAGTCGACAGGGGTCCCAACAATTGGAATCATTAAAAGAATTTCACCATAAACACTCT
GAGGGCCACATGTTCTTTCTGTTAGAATCTGACACCCCTCTCTCTGATTTATGCTGCC
AAGAAGATGCTG. *Ppt1* siRNA single duplex (origene, SR409088),

CTGTTGCAAGGCTTAGATTAGGTTCCAGGCTGACATGACTATGACATGGGCTTGGAAGA
GCTTGTTCTCAAGAATCTGCACTCTGAATAAAGTGGGCAAGGCAATCAGTTAAGAAAAGGTATGATG
TGCCAGTAAACCATTTCTTTAGATATTTGAAGATGTTGCCCTTCTTCTGACCAGAAACACTA
GCTTTCTGCTTTGTCATTATTGCAAGGCGGTCTCTGTGTATAGAGTCTCTCT
Cell Culture

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/L glucose, sodium pyruvate, phenol red, and 10% fetal calf serum (FBS; Sigma-Aldrich, F6178).

BMDMs were isolated as described previously (1) and cultured as above in DMEM in the presence of mouse macrophage Colony stimulating factor (M-CSF), 10 ng/ml. Macrophages Raw 264.7 or BMDMs were M1 polarized with mouse IFN-γ (10 ng/ml) and LPS (100 ng/ml) or M2 polarized with mouse IL-4 (10 ng/ml) and IL-13 (10 ng/ml) for 24 hours. Splenocytes were isolated as described previously (2).

Quantitative Polymerase chain reaction (qPCR) and Primers

Total RNA was isolated by RNA isolation kit (Qiagen, 74134) according to the manufacture's protocol. Complementary DNA (cDNA) was synthesized using iScript Reverse Transcriptase kit with 500 ng of purified RNA as per manufactures protocol (Bio-Rad, 1708890). The qPCR reaction was set up using SYBR green PCR master mix containing 1 μL of cDNA. All measurements were carried out in duplicate, and Hsp90 was used as internal standard for ΔCT calculations. Gene expression analysis was done using following Primers: iNos_For (AGGAGGAGAGAGATCCGATTTAG), iNos_Rev (TCAGACTTCCTGTCTCAGTAG);

Retnla/Fizz1_For (TGCCAATCCAGCTACTATCC), Retnla/Fizz1_Rev (GCAAAGCCACAAGCACAC); Hsp90_For (GGGAGCTCATCTCCAATTCATC), Hsp90_Rev (GTCCTGTTTGCTGGGAATGA); Arg1_For (TACCTCTGCTGGGAAGGAAA), Arg1_Rev (CTGAAGATAGGCCTCCAGA).

Crispr/Cas9 editing
The non-targeting guide RNA (gRNA); TAGCGAACGTGTCCGGCGT and Atg7 guide RNA; AACTCCAACGTCAAGCGGGT sequences for targeting mouse cells were used as described previously (3). The two separate plasmid constructs (1) pCW-Cas9 (Addgene Plasm ID #50661) for the expression of the inducible-hSpCas9 and (2) pLX-sgRNA (Addgene Plasm ID #50662) for the expression of targeting gRNA were used based on the protocol described previously (4). Cloning of the target gRNAs sequence into the pLX-sgRNA vector were performed by three polymerase chain reaction (PCR) extension steps within the XhoI and NheI sites of pLX-sgRNA and were followed by restriction digest and ligation. Sanger sequencing was used to confirm that the Atg7 gRNAs and non-targeting control RNA were correctly cloned in the pLX-sgRNA vector.

B16 cells were first transfected with the pCW-Cas9 vector and selected with puromycin 4ug/ml for 6 days and then transfected with the pLX-sgRNA with containing the Atg7 gRNA and nt gRNA using the Lipofectamine 3000 based on the manufacturer’s instructions. After 48 h incubation, growth medium was changed to selection medium containing blasticidin 6 µg/ml. After blasticidin selection for 12 days, the cells were treated with doxycycline 0.5-1 µg/ml to express Cas9 and induce Atg7 deletion. Doxycycline was replenished every 2 days for 2 weeks, after which the cells are harvested and analyzed by the immunoblotting for Atg7 deletion (Fig. 1a).

Immunoblotting

Whole-cell lysates and lysosomal extracts were immunoblotted as previously described (5, 6).

In vivo studies

Tumor generation, measurement, and harvesting were performed as previously described (7) (9). Briefly, B16 (mouse melanoma) cells were subcutaneously injected (0.5x10^5) with an equal volume of matrigel (Corning, 354234) in the Right flank of C57BL6J mice. Everyday injection of HCQ (60 mg/Kg) and every other day of anti-PD-1 Ab (200 µg) commenced at the tumor size of 50 mm³. BRaf^CA, Pten^loxP, Tyr::CreER^T2 mice were treated topically on the back with 4-HT to elicit BRaf^V600E and to silence Pten expression. Tumors were measured using electric calipers.
Tumor volume was calculated as $L \times W^2 \times 0.5$. In all animal experiments t-test or a t-test for the unequal variance were used to test the hypothesis that the addition of HCQ to anti-PD-1 Ab is significantly different compared to anti-PD-1 Ab + Veh.

**Tumor digestion**

Tumor was excised and digested with tumor digestion kit according to the manufacture’s protocol.

**Flow cytometry and IHC**

1x10^6 cells from digested tumor were stained with antibodies (please see Supplemental Figure S2) Table 1. Stained samples were acquired on LSR and the gating strategy was followed as in Supplemental figure S4A and S5A. Mouse tumor IHC was performed as per standard protocol.

**T cell priming and percent cytotoxicity**

0.5x10^5 B16 tumor cells were cultured and irradiated with 25 gray (gy) x-rays. Splenocytes were then cultured with (primed) or without (unprimed) irradiated B16 cells in the presence of Interleukin-2 (IL-2) (5 IU/ml) and co-cultured for 48 hours. Splenocytes cultured with concanavalin A (10 µg/ml) was used as a nonspecific T cell priming control. Priming was confirmed by IFN-γ, ELISA of the supernatant. Primed splenocytes were then co-cultured with freshly cultured B16 cells with various target (B16) to effector (splenocytes) ratios. The B16 cell death associated lactate dehydrogenase (LDH) release and then % cytotoxicity was measured according to the manufacture’s protocol.

**Macrophage conditioned media**

Macrophages conditioned media (MCM) was generated by treating M2 polarized Raw 264.7 macrophages with vehicle or DC661 for 6 and 24 h. Supernatant was spun at 2000 rpm for 3 min
to remove any viable or dead macrophages and then MCM was added to fresh B16 cells co-
cultured with Primed or unprimed splenocytes. LDH release and % cytotoxicity was measured.

**Proteomics and secretome analysis**

Macrophage conditioned media (MCM) from M2 polarized macrophages grown in 0.01% serum was centrifuged to remove cellular debris. The supernatant was passed through a 0.2 µm filter, concentrated 50-fold using an Amicon Ultra 3K membrane, electrophoresed for 0.5 cm into an SDS-PAGE gel and stained with colloidal Coomassie. The entire stained region was excised, digested with trypsin, and analyzed by LC/MS-MS on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled with a Nano-ACQUITY UPLC system using a 245 minute gradient and acquisition parameters as previously described (24). RAW files were searched using MaxQuant 1.5.3.30 using default parameters and peptide sequences were identified against a mouse UniProt database (October 2019) using full tryptic specificity, up to 2 missed cleavages, fixed modification of carbamidomethyl (Cys), and variable Met oxidation and protein N-terminal acetylation. An in house common contaminant database comprised of trypsin, keratins, bovine proteins found in serum, and mycoplasma proteins was appended to the mouse sequence database. Protein and peptide false discovery rates were set at 1%. Proteins identified by at least two peptides were analyzed using label free quantitation to determine the ratio of protein intensity in HCQ or DC661 treated MCM verses control.

**Study Approval**

All animal experiments were performed in accordance with the protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

**Statistical analysis**

For continuous variables, a two-sample t-test was used to test the primary hypothesis that there was a significant difference between the two experimental groups receiving either HCQ and anti-
PD-1 Ab or anti-PD-1 Ab + Veh. For experiments comparing more than two groups, continuous variables were analyzed using one-way analysis of variance with adjusted p-values using Dunnett’s procedure when each experimental group was compared to control or Tukey’s procedure when all pairwise comparisons were considered. For relative expression, a one-sample t-test was used to test the hypothesis that mean differences were different from 1 with adjusted p-values computed using the Holm-Bonferroni procedure. For survival times, an exact log-rank test was used to compare the survival curves between the HCQ and anti-PD-1 Ab or anti-PD-1 Ab + Veh. groups. Growth rates (mm³/day) were computed for each mouse using linear regression of the natural logarithm of tumor volume on time; the estimated slopes for the HCQ and anti-PD-1 Ab or anti-PD-1 Ab + Veh. groups were analyzed using a two-sample t-test. A p-value less than 0.05 was considered significant. P-values presented in the figures are for the test of the hypothesis that the expected mean when HCQ is added to anti-PD-1 Ab is significantly different than the expected mean for anti-PD-1 Ab + Veh. Adjusted p-values are indicated as * when the adjusted p<0.05 and as ^ when the adjusted p<0.10. All analyses were done using SAS/STAT software, version 9.4 of the SAS system for Windows.

Acknowledgments:

We thank the members of Constantinos Koumenis lab in the Department of Radiation Oncology, for experimental support.

Author Contributions:

supervised experiments and analyzed the data. G.S., D.S., D.G., P.G. and R.A. wrote the manuscript with assistance from all the other authors.

Disclosure of Potential Conflicts of Interest: RA and JW are co-founder of Pinpoint Therapeutics, and inventor on patents covering dimeric chloroquine compounds. RA is a consultant for Sprint Biosciences and Immunacel, and Array Pharmaceuticals.

Funding: This work was supported by NIH grants R01CA169134; P01CA114046; 1P01CA165997-02; P30 CA016520; SPORE P50 CA174523; 1R01CA198015. This work was also supported by the Penn-Incyte Alliance and the Tara Miller Foundation.

References


Figure 1. PPT1 inhibitor HCQ enhanced the anti-tumor efficacy of anti-PD-1 Ab in a mouse melanoma model. (A) Tumor growth curve, 5x10^4 B16 cells were injected in the flank of C57Bl6/J mice, after tumors reached a size of 50 mm³ mice were randomized to cohorts of n=5 mice each. Mice were treated with either IgG control 200 ug or anti-PD-1 Ab 200 μg every other day, and either water or HCQ 60 mg/kg i.p. every day and tumors were measured every day. (B) Picture of excised B16 tumors (C) Final tumor weight (D) Immunoblot analysis of autophagy (LC3B-I/II, SQSTM1/p62) and apoptosis markers (caspase 3 and caspase 7) with quantification of the bands. (E) Kaplan-Meier Survival curve of mice treated as above in a new experiment. The average and standard error of the mean was calculated for each treatment cohort. A p-value is presented for the test of the hypothesis that the addition of HCQ to anti-PD-1 Ab is significantly different compared to anti-PD-1 Ab + Veh; * indicates adjusted p<0.05 and ^ indicates adjusted p<0.10 testing the hypothesis that the mean relative risk is different from 1.
Figure 2. The effects of PPT1 inhibition are not recapitulated by Ulk1, Pik3c3 and Atg7 KD or Atg7 KO and HCQ does not impair CD8+ T cell mediated killing. (A) Schematic of priming and co-culture experiments to measure antigen-specific T cell killing in vitro. (B) 100-%LDH from the co-culture of primed or unprimed splenocytes with live B16, in the presence or absence of HCQ (10 µM) each experiment was performed in triplicates and results were reproduced with 3 independent experiments. Concanavalin A was used as non-specific splenocyte priming agent. (C) 100-% LDH measurement in primed splenocytes co-cultured with B16 +/- indicated treatments. (D) Immunoblots confirming the B16 Ulk1, Pik3c3 and Atg7 KD status. (E) Dot plot representing ELISA performed for the measurement of splenocyte secreted IFN-γ upon co-culturing with B16 with Ulk1, Pik3c3 and Atg7 KD conditions each experiment was performed in triplicates and the results were reproduced by 3 independent experiments. (F) Immunoblot
confirming the B16 *Ppt1* KD status. (G) Dot plot representing IFN-γ ELISA in *Ppt1* KD B16 co-culture as above in E for 3 independent experiments. (H) Measurement of % T cell mediated cytotoxicity of B16 cells in *Ulk1, Pik3c3, Atg7* and *Ppt1* condition. (I) Immunoblot confirming the B16 *Atg7* KO status. (J) Irradiated B16-primed splenocytes or purified splenic CD8+ T cells were co-cultured with B16 WT *Atg7* or B16 *Atg7* KO cells and the percentage proliferation was measured by performing 3 independent experiments. (K) 5x10^5 B16 Cas9 control or B16 *Atg7*KO cells were injected into the flanks of C57Bl6J mice. After tumors reached a size of 50 mm^3^ mice were randomized to cohorts of n=5 mice each and B16 cas9 control tumors were treated with IgG control + vehicle, Anti-PD-1 Ab (200 µg i.p. every other day) + vehicle, IgG + HCQ (60 mg/kg i.p. daily), or the combination. B16 *Atg7* KO tumors were treated with either IgG control or anti-PD-1 Ab. The average growth rate +/- S.E.M. is shown. (L) Tumors and spleens were harvested from the experiment in B, on day 8 of treatment. The percent of CD8+ T cells in CD45+ cells in spleen and tumor are shown.
Figure 3. PPT1 inhibition induces a change in macrophage polarization that favors antitumor immunity. (A) Dot plot for the qPCR expression in mouse macrophage Raw 264.7 cells polarized to an M2 phenotype following treatment with HCQ 10 μM or DC661 0.6 μM at the indicated time points (in hours) showing the results of 4 independent experiments. (B) Bright field images of Raw 264.7 polarized to an M2 phenotype treated with HCQ or DC661 M1 phenotype control (IFN-γ + LPS) is included. M2 cells have a round morphology whereas drug treated M2
cells take on an elongated morphology with multiple pseudopodia typical of M1 macrophages (positive control), images were taken at 10X magnification. (C) Expression of M2 and M1 markers in mouse bone marrow derived macrophages (BMDM)s treated with HCQ or DC661 as in figure a, each result was reproduced by 3 independent experiments. (D) Dot plot showing qPCR expression of iNOS and RETNLA/FIZZ1 following Ppt1 knockdown in Raw 264.7 cells polarized to an M2 phenotype and each result was reproduced by 5 independent experiments; immunoblot showing the knockdown status of Ppt1 protein. (E) qPCR expression of M1 and M2 markers in Ulk1, Pik3c3 and Atg7 KD conditions and results were reproduced with 3 independent experiments; immunoblots showing the knockdown status of Ulk1, Pik3c3, Atg7 protein and expression of LC3, p62. (F) Schema of experimental setup and cytotoxicity elicited by primed splenocytes with or without exposure to macrophages conditioned media (MCM) collected from RAW 264.7 macrophages treated with control HCQ or DC661 for 24 hours. (G) Immunophenotyping for M1/M2 ratio of tumor associated macrophages (TAMs) and % PMN-MDSC in B16 melanoma tumors after 8 days of treatment. Mean and SEM are representative of 4 to 5 replicates and the experiment was repeated at least 3 times. A p-value is presented for the test of the hypothesis that the addition of HCQ to anti-PD-1 Ab is significantly different compared to anti-PD-1 Ab + Veh; * indicates an adjusted p<0.05 testing the hypothesis that each experimental group is different from control. Scale bar: 100 μm.
Figure 4. HCQ and anti-PD-1 Ab combination impairs tumor growth and reduces the MDSCs infiltration in Braf<sup>CAPtenfloxfloxTyr::CreERT2</sup> melanoma model. (A) Topical 4-HT was applied on the back to elicit spontaneous melanoma growth (n=4 per treatment), and once tumors were palpable treatment as in Fig. 1 was started. (B) Representative images of mice (C) Representative images of IHC staining of tumor against Ly6C/Ly6G (MDSC marker) at 10X magnification (inset of each image is 2.5X magnified). (D) number of Ly6C/Ly6G positive cells per high powered field. (E) immunophenotyping of M-MDSCs in tumor. (F) Model of lysosomal inhibitor action in combination with anti-PD-1 Ab against TAM/MDSC, T cells and tumor cells in tumor microenvironment. A p-value is presented for the test of the hypothesis that the addition of HCQ to anti-PD-1 Ab is significantly different compared to anti-PD-1 Ab + Veh.
Figure 5. PPT1 acts as a molecular switch and its inhibition results in calcium dependent p38 phosphorylation and macrophage polarization. (A) Confocal microscopy of Raw264.7 macrophages for staining calcium by Fluo-4, AM dye in PPT1 inhibitors HCQ and DC661 treated cells, images were taken at 40X under Olympus IX71 confocal microscope. (B) Immunoblots showing p-p38 and total p38 in DC661, HCQ, HDSF and LPS treated Raw264.7 macrophages. (C) Immunoblots for p-p38 and p38 in Ppt1 KD condition in Raw264.7 macrophages. (D) Immunoblots for p-p38 in HCQ or DC661 with co-treatment of calcium chelator BAPTA-AM. (E) Immunoblots for p-p38 in HCQ and DC661 or co-treatment with calmodulin inhibitor W7. (H) Immunoblots for p-p38 in HCQ, DC661 or TRPML-1 agonist MK6-83 or co-treatment with TRPML-1 inhibitor verapamil. I, Immunoblots for p-p38 in HCQ, DC661 or TRPML-
agonist MK6-83 or co-treatment with PIKfyve inhibitor vacuolin-1. (J) Immunoblot for p-p38 in DC661 treated cells or co-treatment of ER calcium channel inhibitor ryanodine or mitochondrial sodium-calcium exchanger inhibitor CGP37157. Immunoblot for p-p38 in TRPML-1 agonist or co-treatment of ryanodine or CGP37157 in macrophages. * indicates p<0.05. Scale bar: 100 μm
Figure 6. PPT1 inhibition stimulates IFN-β secretion by macrophages via activation of cGAS/STING/TBK1 pathway and enhances T cell anti-tumor activity. (A) Relative MS signal of proteins in control, HCQ or DC661 treated MCM that showed a five-fold increase and p-value < 0.05 in HCQ treated MCM relative to control. (B) Dot plot representing ELISA performed for the measurement of IFN-β in HCQ or DC661 treated macrophage conditioned media, each result was obtained in duplicates and the results were reproduced with 3 independent experiments. (C) Immunoblots showing cGAS, STING and p-TBK1 protein status in macrophages treated with HCQ, DC661 or DMXAA for the time indicated. (D) Immunoblots for cGAS, STING and p-TBK1
proteins in Ppt1 KD macrophages. (E) Dot plots representing ELISA performed for the measurement of IFN-β in HCQ or DC661 treated or STING inhibitor C-176 or TBK1 inhibitor GSK8612 co-treated macrophage conditioned media each result was obtained in duplicates and the results were reproduced with 3 independent experiments. (F) Immunoblot showing the status of p-STAT-1 in Isotype or anti-IFN- β neutralizing antibody in the presence or absence of recombinant IFN- β in mouse splenocytes. (G) % cytotoxicity elicited by primed splenocytes with exposure to macrophages conditioned media (MCM) collected from RAW 264.7 macrophages treated with control, HCQ or DC661 along with the administration of isotype or anti-IFN- β neutralizing antibody as indicated, each result was obtained in duplicates and the results were reproduced with 3 independent experiments. (H) Schematic showing the PPT1 inhibition results in reduced MDSCs tumor infiltrations and macrophage M2 to M1 switching. Polarized macrophages upon PPT1 inhibition secrete IFN-β which enhances the CD8 T cells mediated tumor cells killing.