Dimeric quinacrinines as chemical tools to identify PPT1, a new regulator of autophagy in cancer cells

Michael C. Nicastri, Vito W. Rebecca, Ravi K. Amaravadi, and Jeffrey D. Winkler

ABSTRACT
DQ661 is a novel dimeric quinacrine that affects multiple lysosomal functions (autophagy and macropinocytosis) and mTORC1 (mechanistic target of rapamycin) activity by specifically targeting protein-palmitoyl thioesterase 1 (PPT1). DQ661 has in vivo activity in immunocompetent mouse models of cancer, and constitutes a new tool compound for the study of lysosomal function in cancer and therapeutic resistance.

Preclinical studies that demonstrate targeting the lysosome can improve anti-cancer therapy have led to >20 phase I/II clinical trials combining anti-cancer agents with the lysosome inhibitor hydroxychloroquine (HCQ). While targeting the lysosome can produce measurable autophagy inhibition, HCQ does not consistently inhibit autophagy in patient tumors. Therefore, more potent lysosome inhibitors are urgently needed.

Previously we reported Lys05, a dimeric chloroquine possessing an increased ability to inhibit autophagy-lysosome function in vivo relative to HCQ. Structure activity relationship (SAR) studies of Lys05 demonstrated that dimeric inhibitors possess increased anti-autophagy and anti-cancer activity compared to monomeric chloroquine. Building on this finding, our laboratory designed a series of dimeric inhibitors derived from other quinoline-based compounds (Fig 1A). Dimerization of quinacrine, primaquine, and mefloquine all resulted in enhanced anti-cancer activity relative to the respective monomeric parent compounds, with the greatest enhancement of activity observed on dimerization of quinacrine (DQ). We next synthesized an expanded DQ library in which the polyamine linker was varied in both length and substitution.

Significant differences were observed in the biological properties of the resulting DQs as a function of the substitution of the central nitrogen (N-H vs. N-Methyl). Unmethylated compounds produced double stranded DNA damage and induced autophagy. In contrast, methylated compounds co-localized more efficiently with lysosomes, producing no DNA damage and inhibiting autophagy. DQ661 emerged as the most potent inhibitor of autophagy and the best performing compound in assays measuring anti-cancer activity. We leveraged the potency of DQ661 to determine the molecular target for these agents.

A photoaffinity pull-down experiment was utilized to identify protein-palmitoyl thioesterase 1 (PPT1), a lysosomal thioesterase, as the specific binding partner of DQ661 (Fig 1B). In this experiment, a benzophenone moiety attached to DQ661 (DQ661 photoprobe) was covalently linked to potential target proteins on irradiation. The experiment was repeated in the presence of free DQ661 (competition experiment). Proteomic studies performed on pulled down photoprobe-protein conjugates established that PPT1 was the only lysosomal protein that was pulled down in a DQ661-specific manner. Confirmation of drug-protein binding was established by differential scanning calorimetry. Incubation of recombinant PPT1 with DQ661 caused a significant shift in the observed protein melting temperature. We also demonstrated that treatment of cancer cells with DQ661 inhibited PPT1 enzymatic activity. Genetic silencing of PPT1 phenocopied the effects of DQ661 on autophagy-lysosome function in cancer cells, further validating PPT1 inhibition as a novel strategy to impair lysosomal catabolism.

The identification of PPT1 as a new lysosomal target in cancer represents an innovative, targeted approach to inhibiting autophagy. The current landscape of autophagy inhibitors can be parsed into those that target early- or late-stage autophagy. Early autophagy inhibitors, including Spautin-1 and SBI-0206965, target proteins involved in the initiation, formation, and elongation of autophagosomes. However, the existence of non-canonical forms of autophagy reveals that strategies which target a canonical autophagy protein may be circumvented. As canonical and non-canonical autophagy both rely on the lysosome for degradation and recycling of nutrients, the lysosome provides the optimal target. The identification of PPT1 provides a lysosomal...
target for which more potent lysosome inhibitors can be designed.

To understand how DQ661-mediated inhibition of PPT1 could impact intracellular signaling pathways, Reverse Phase Protein Array (RPPA) studies were performed. RPPA studies identified mTORC1 (mechanistic target of rapamycin) signaling as the most significantly impacted pathway in melanoma cells treated with DQ661. This effect of DQ661 on mTORC1 is unique amongst lysosomal inhibitors, including the vacuolar (v)-ATPase inhibitor bafilomycin and the acid sphingomyelinase inhibitor siramesine. For its full activation, mTORC1 localizes to the lysosomal surface in an amino acid-dependent manner, in proximity to its activator protein Rheb (Ras homolog enriched in brain). The localization of mTORC1 to the lysosome occurs through critical interactions between the v-ATPase and the Ragulator machinery.8 Recently, neuronal cells deficient in PPT1 displayed mislocalized v-ATPase, leading to lysosomal deacidification.9 In cancer cells, targeting PPT1 with DQ661 or siRNA both resulted in the mislocalization of v-ATPase from the lysosome. V-ATPase mislocalization has two major consequences: 1) lysosomal deacidification leading to the blockade of autophagic flux, and 2) disruption of the physical interaction between v-ATPase and Ragulator, a key component for the lysosomal recruitment of mTOR (Fig 1C). Thioesterase mimic n-tert-butyl-hydroxylamine rescued autophagy and mTORC1 inhibition by DQ661, providing further support that PPT1 is the target of DQ661. Experiments are underway in our laboratory to determine if v-ATPase mislocalization is the sole mechanism for the observed effects of PPT1 inhibition on both autophagy and mTOR signaling.

Despite reports demonstrating that mTORC1 serves a critical role in therapeutic resistance in cancer patients, specific mTORC1 inhibitors (allosteric and catalytic) have largely failed to provide meaningful efficacy in the clinic.10 Loss of mTORC1 activity leads to cytoprotective induction of lysosomal catabolism (macropinocytosis and autophagy), often rendering single-agent mTORC1 inhibitors ineffective. Inhibition of PPT1 both functionally inactivates mTORC1 by disrupting its lysosomal localization, and inhibits the induction of lysosomal catabolism. The utility of combining DQ661 with existing mTORC1 inhibitors may further improve efficacy by simultaneously targeting different components of mTORC1 activation dynamics, and preventing acquired drug resistance.

We have identified PPT1 as a regulator of two key processes driving cancer aggressiveness; mTORC1 and autophagy. The full role of PPT1-dependent depalmitoylation in oncogenesis

Figure 1. Schematic summary of the invention of DQ661 (a dimeric quinacrine autophagy/lysosome inhibitor), the identification of Protein Palmitoyl Thioesterase 1 (PPT1), and their influence on lysosomal biology. A) Depicts the chemical strategy in the design and synthesis of lysosomal inhibitor DQ661. Newly described compounds are abbreviated with the identity of their parent heterocycle: dimeric chloroquine (DC), dimeric quinacrine (DQ), dimeric primaquine (DP), and dimeric mefloquine (DM). After the two letter code, the length of the polyamine linker is specified by the first two numbers, and followed by a zero (Unmethylated) or one (methylated) B) Outline of a photoafinity experiment which identifies PPT1 as a protein binding partner of DQ661 C) Schematic demonstrating how DQ661-mediated inhibition of PPT1 results in the disruption of physical interaction of key protein complexes involved in lysosomal acidification and mTORC1 (mechanistic target of rapamycin) activation.
remains to be delineated. Beyond the disruption of nutrient scavenging via macropinocytosis observed by DQ661-mediated inhibition of PPT1, other metabolic consequences remain to be elucidated. Investigations into the antimalarial activity of DQ661 and its analogs are also currently under way. SAR studies directed toward the development of more potent anti-cancer agents and PPT1 inhibitors based on the structure of DQ661 are in progress in our laboratories.

**Conflict of interest statement**

RA and JW are inventors on 3 patent applications related to this work. One patent has been licensed to a biotech company to promote clinical development of Lys05 derivatives.

**Funding**

This work was entirely supported by NIH grants R01CA16934; P01 CA114046; P30 CA016520; SPORE P50 CA174523; 1R01CA198015; CA016672; P30CA010815.

**References**

1. Ma XH, Piao SF, Dey S, McAfee Q, Karakousis G, Villanueva J, Hart LS, Levi S, Hu J, Zhang G, et al. Targeting ER stress-induced autophagy overcomes BRAF inhibitor resistance in melanoma. The Journal of clinical investigation. 2014;124(3):1406–1417.

2. Rangwala R, Chang YC, Hu J, Algazy KM, Evans TL, Fecher LA, Schuchter LM, Torigian DA, Panosian JT, Troxel AB, et al. Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. Autophagy. 2014;10(8):1391–402.

3. Mahalingam D, Mita M, Sarantopoulos J, Wood L, Amaravadi RK, Davis LE, Mita AC, Curiel TJ, Espitia CM, Nawrocki ST, et al. Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy. 2014;10(8):1403–14.

4. McAfee Q, Zhang Z, Samanta A, Levi SM, Ma XH, Piao S, Lynch JP, Uehara T, Sepulveda AR, Davis LE, et al. Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(21):8253–8. [Pmc3361415]

5. Rebecca VW, Nicastri MC, McLaughlin N, Fennelly C, McAfee Q, Ronghe A, Nofal M, Lim CY, Witze E, Chude CI, et al. A unified approach to targeting the lysosome’s degradative and growth signaling roles. Cancer Discov. 2017.

6. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, Cai Y, Norberg HV, Zhang T, Furuya T, et al. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell. 2011;147(1):223–34. [Pmc3441147]

7. Codogno P, Mehrpour M, Proikas-Cezanne T. Canonical and non-canonical autophagy: variations on a common theme of self-eating? Nat Rev Mol Cell Biol. 2012;13(1):7–12.

8. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)−ATPase. Science. 2011;334(6056):678–83. [Pmc3211112]

9. Bagh MB, Peng S, Chandra G, Zhang Z, Singh SP, Pattabiraman N, Liu A, Mukherjee AB. Misrouting of v-ATPase subunit V0a1 dysregulates lysosomal acidification in a neurodegenerative lysosomal storage disease model. Nature communications. 2017;8(14612). [Pmc5344305]

10. Klempner SJ, Myers AP, Cantley LC. What a tangled web we weave: emerging resistance mechanisms to inhibition of the phosphoinositide 3-kinase pathway. Cancer Discov. 2013;3(12):1345–54. [Pmc3864542]