A Unified Approach to Targeting the Lysosome’s Degradative and Growth Signaling Roles

Vito W. Rebecca1, Michael C. Nicastri2, Noel McLaughlin2, Colin Fennelly1, Quentin McAfee1, Amruta Ronghe3, Michel Nofal4, Chun-Yan Lim5, Eric Witze6, Cynthia I. Chude1, Gao Zhang3, Gretchen M. Alicea3, Shengfu Piao1, Sengottuvelan Murugan1, Rani Ojha1, Samuel M. Levi2, Zhi Wei7, Julie S. Barber-Rotenberg8, Maureen E. Murphy3, Gordon B. Mills9, Yiling Lu9, Joshua Rabinowitz4, Ronen Marmorstein6, Qin Liu3, Shujing Liu10, Xiaowei Xu10, Meenhard Herlyn3, Roberto Zoncu5, Donita C. Brady6, David W. Speicher3, Jeffrey D. Winkler2,11, and Ravi K. Amaravadi1,11
Lysosomes serve dual roles in cancer metabolism, executing catabolic programs (i.e., autophagy and macropinocytosis) while promoting mTORC1-dependent anabolism. Antimalarial compounds such as chloroquine or quinacrine have been used as lysosomal inhibitors, but fail to inhibit mTOR signaling. Further, the molecular target of these agents has not been identified. We report a screen of novel dimeric antimalarials that identifies dimeric quinacrines (DQ) as potent anticancer compounds, which concurrently inhibit mTOR and autophagy. Central nitrogen methylation of the DQ linker enhances lysosomal localization and potency. An in situ photoaffinity pulldown identified palmitoyl-protein thioesterase 1 (PPT1) as the molecular target of DQ661. PPT1 inhibition concurrently impairs mTOR and lysosomal catabolism through the rapid accumulation of palmitoylated proteins. DQ661 inhibits the in vivo tumor growth of melanoma, pancreatic cancer, and colorectal cancer mouse models and can be safely combined with chemotherapy. Thus, lysosome-directed PPT1 inhibitors represent a new approach to concurrently targeting mTORC1 and lysosomal catabolism in cancer.

SIGNIFICANCE: This study identifies chemical features of dimeric compounds that increase their lysosomal specificity, and a new molecular target for these compounds, reclassifying these compounds as targeted therapies. Targeting PPT1 blocks mTOR signaling in a manner distinct from catalytic inhibitors, while concurrently inhibiting autophagy, thereby providing a new strategy for cancer therapy.

Cancer Discov; 7(11): 1–18. © 2017 AACR.
goal, we modified the Lys05 structure, ultimately yielding a series of potent dimeric quinacridines (DQ). We find that methylation of the central nitrogen of the linker affects DQ subcellular localization and enhances DQ function against autophagy. This approach has led to the identification of a class of compounds that can be targeted specifically to the lysosome. Using these more potent and lysosome-specific inhibitors, we have identified the molecular target of DQ as palmitoyl-protein thioesterase 1 (PPT1), resulting in the impairment of mTOR signaling via disruption of the interaction between mTORC1 and its master activator RHEB, even in the setting of AA-replete medium. The concurrent impairment of mTORC1 and lysosomal catabolism, one of the key resistance mechanisms to existing mTOR inhibitors, leads to a more complete abrogation of protein translation and anticancer activity relative to what is achievable with allostERIC or catalytic mTORC1 inhibitors. Our findings reveal an unanticipated role of PPT1 in regulating the triamine linker of Lys05 using the naturally occurring lysosomal catabolism, one of the key resistance mechanisms to existing mTOR inhibitors. Our findings reveals an unanticipated role of PPT1 in regulating

RESULTS

DQ Has Superior Anticancer Efficacy among Dimeric Antimalarials

In an effort to improve the activity of Lys05, we extended the triamine linker of Lys05 using the naturally occurring triamine spermidine. The anticancer activity of the resulting compound, DC340, the name of which was derived as described below, was assessed in BRAF-mutant melanoma and KRAS-mutant pancreatic cancer cell lines, as cancers driven by these oncogenes depend on autophagy/lysosome function (12, 13). DC340 inhibited the growth of a panel of melanoma and pancreatic cancer cell lines significantly better than Lys05 (Fig. 1A and B). We next developed a series of dimeric compounds linked by the spermidine linker, whereby the 7-chloroquinolinoquinone heterocycles of CQ were replaced with the heterocyclic rings present in other antimalarials, such as primaquine (PQ), melquine (MQ), and quinacrine (QN; Fig. 1A). We describe these resulting novel structures as dimeric CQ (DC), dimeric MQ (DM), dimeric PQ (DP), or dimeric QN (DQ), followed by three digits (m, n, R), where m and n signify the number of carbons to the left and right of the central nitrogen of the triamine linker, respectively, and the R signifies whether the central nitrogen of the linker is methylated (1) or not (0; Fig. 1A). Antiproliferative activity was improved between 3- and 10-fold for each dimeric compound that contained CQ, MQ, or PQ, relative to its monomeric parent. In contrast, dimerization of QN resulted in a 60-fold improvement in antiproliferative activity relative to monomeric QN (Fig. 1C).

We next generated a focused library of DQs with triamine linkers of increasing length, ranging from 2 to 11 carbons between the 9-aminoacridine and the central nitrogen of the triamine linker. Both unmethylated (R = 0) and methylated (R = 1) derivatives of each analogue were prepared to determine the role of central nitrogen methylation on biological activity. Treatment of A375P and PANC1 cells with this DQ library further established the superior antiproliferative potency of DQs compared with their monomeric counterpart (QN; Fig. 1D; Supplementary Fig. S1A). Although DQ221, the acridine dimer with the same linker as Lys05 (DC221 using this nomenclature system), showed the same potency as its corresponding monomer with no benefit from dimerization, a clear relationship between linker length and potency emerged among the DQs with increasing linker length, whereby DQs with 3 to 6 carbons between linker nitrogens possessed significant antiproliferative potency. Further extension of the distance between the two QN heterocycles resulted in incrementally decreased antiproliferative activity (Fig. 1D; Supplementary Fig. S1A). Pharmacologic agents that specifically inhibit autophagy, such as Spautin-1, which promotes degradation of Beclin (14), and SBI-0206965 (SBI), an unc-like kinase 1 (ULK1) inhibitor (14, 15), exhibited significantly less potency relative to DQs (Fig. 1D; Supplementary Fig. S1A). The compounds in the series DQ330–DQ661 produce significantly greater long-term growth suppression than monomeric QN and DQ221 (Supplementary Fig. S1B). Unlike Spautin-1 and SBI, which did not induce appreciable levels of apoptosis, the majority of DQs trigger significantly greater levels of apoptosis, which directly correlates with increasing linker length, relative to monomeric QN (Fig. 1E).

Central Nitrogen Methylation Directs Subcellular Localization of DQs

We next interrogated the specificity of DQs as lysosomal inhibitors, as the parent monomer, QN, both binds to DNA in the nucleus and accumulates in the lysosome (16). The inherent fluorescence of DQs was exploited to study their subcellular localization. The most potent longer-linked DQs (DQ550, DQ551, DQ660, and DQ661) fluoresce in both the red and green channels, under both neutral (pH = 7) and acidic (pH = 4) conditions (Fig. 2A). Under neutral conditions, methylated DQs demonstrated increased green fluorescence compared with unmethylated DQs. However, there were no significant differences in red fluorescence observed between unmethylated and methylated DQs. Acidic conditions led to partial quenching of green fluorescence, but had minimal effects on red fluorescence in both methylated (DQ551 and DQ661) and unmethylated (DQ550 and DQ660) DQs. These findings indicate that red fluorescence is more effective to assess lysosomal localization of these compounds. Surprisingly, each unmethylated DQ (DQ550 and DQ660) displayed no detectable red fluorescence, whereas each methylated DQ (DQ551 and DQ661) possessed strong red fluorescence in the lysosomal compartment (Fig. 2B; Supplementary Fig. S2A). No colocalization with mitochondria was observed for DQ551 or DQ661 (Supplementary Fig. S2B). To rule out the possibility that the addition of a methyl group to the central nitrogen was simply increasing the basicity of the DQ, thereby trapping the methylated derivatives in the acidic lysosome, the pKₐ values of each DQ were calculated (Supplementary Table S1). This analysis demonstrated that the pKₐ of each pair of unmethylated and methylated DQ did not differ significantly, and therefore differential basicity could not account for the difference in subcellular localization. Having established the role of central nitrogen methylation as a determinant of lysosomal localization, we next characterized how DQs affect autophagy. Changes in autophagic vesicle (AV) levels were interrogated by measuring ATG8/LC3B (LC3I, LC3II...
Dimeric Quinacrine Identify New Lysosomal Target in Cancer

Figure 1. DQs have superior anticancer efficacy among dimeric antimalarials. A, Chemical structures of dimeric antimalarials. Ar, aromatic ring.

B, Cells were treated with compounds shown (2 weeks, 3 μmol/L) in colony formation assays. C, Calculated log IC50 values from 72-hour MTT assays in A375P (melanoma) cells treated with compounds shown. D, Calculated log IC50 values of indicated compounds (72 hours, 1 nmol/L–30 μmol/L) from MTT assays in A375P cells.

E, PANC1 cells were treated as indicated (24 hours, 3 μmol/L), stained with Annexin V, and analyzed using flow cytometry. B–D, For all graphs, mean ± SD for N = 3 independent experiments are presented; *, P < 0.05.

hereafter) protein levels, as LC3II is the most reliable protein marker of completed autophagosomes (17). Increasing DQ linker length was associated with increasing LC3II/LC3I ratios (Fig. 2C), reflecting an accumulation of AVs. Interestingly, there was also a relationship between central nitrogen methylation and LC3II/LC3I ratios, in which compounds with central nitrogen methylation (DQXX1) had a significantly higher LC3II/LC3I ratio relative to their unmethylated (DQXX0) counterparts. Due to the inherent fluorescence of QN, spectral overlap with mCherry-eGFP-LC3 expressing cells (18) prevented the use of this approach to characterize autophagic flux. The effects of these compounds on autophagic flux were therefore determined by a bafilomycin (baf) clamp experiment (19). Treatment with DQs lacking central nitrogen methylation (DQXX0) resulted in a further increase in the LC3II/LC3I ratio in baf-treated cells compared with control...
Figure 2. Central nitrogen methylation status directs effects upon autophagy, induction versus inhibition. A, Fluorescence spectroscopy emission spectra are shown (excitation 424 nm). B, Fluorescent microscopy of PANC1 (pancreas cancer) cells treated as indicated (6 hours, 3 μmol/L) and costained with LysoTracker deep red (shown green); arrows, colocalization of DQ compound (red) with LysoTracker (green). Scale bar, 50 μM. C, LC3B immunoblot in lysates from C8161 (melanoma cells) treated as indicated (4 hours, 3 μmol/L). D, Bafilomycin clamp (100 nmol/L) to measure autophagic flux was performed on A375P cells treated with DQ compounds (4 hours, 3 μmol/L). Lysate was immunoblotted for LC3B. E, PANC1 cells were treated with compounds shown (6 hours, 3 μmol/L). Lysates were immunoblotted and change in p62 densitometry levels were quantified and are depicted in the graph to the right; *, P < 0.05. U, unmethylated compounds; M, methylated compounds. F, A375P cells were treated with DQ660 or DQ661 (6 hours, 3 μmol/L), and lysosomal and cytosolic fractions were subsequently isolated. Lysates were immunoblotted for LAMP2 and Tubulin (left). Bio-orthogonal fluorescence of DQ660 and DQ661 was measured on a fluorescent plate reader to quantify the lysosomal quantity of DQ660 and DQ661. WC, whole cell fraction; L, lysosome-enriched fraction. In all cases, *, P < 0.05.
Dimeric Quinacridines Identify New Lysosomal Target in Cancer

DQs Cause Either Significant DNA Damage or Lysosomal Membrane Permeabilization Depending on the Central Nitrogen Methylation Status

We next determined whether DQs cause DNA damage, because QN is known to intercalate DNA (20). A striking relationship between nitrogen methylation and DNA damage emerged, whereby unmethylated DQs induced significantly greater levels of DNA damage relative to their methylated counterparts (Fig. 3A). Furthermore, the difference in DNA damage induced by unmethylated DQs versus the corresponding methylated analogues increased with increasing linker length for each pair of compounds. The integrity of the lysosomal membrane was interrogated by detection of lectin galactoside binding soluble 3 (LGALS3)/galectin-3 localization, which was recently demonstrated to rapidly translocate to damaged lysosomes (21). When lysosomes are healthy, galectin-3 is uniformly dispersed throughout the cell. However, when the lysosome is damaged, galectin-3 rapidly localizes to the site of damaged lysosomes, appearing as puncta. Only methylated DQs (DQXX1) caused lysosomal membrane permeabilization (LMP), as seen by the formation of galectin-3 puncta (Fig. 3B). The differential ability to induce LMP was further demonstrated by the observation of mature cathepsin D in the extralysosomal fraction of cells treated with DQ661 (Fig. 3C). DQ661 emerged as the lead lysosomal inhibitor in this series of compounds, capable of most potently inducing DNA damage-independent apoptosis, blocking autophagic flux, and triggering LMP. To determine the underlying mechanism of action of DQ661, treated cells were assayed for >290 proteins or phosphoproteins spanning key signaling programs commonly perturbed in cancer cells using reverse phase protein arrays (RPPA). The proteins most significantly and reproducibly inhibited by DQ661 were downstream of mTORC1 signaling (Fig. 3D; Supplementary Fig. S3A). These included reduced phosphorylation of PRAS40, p70S6K, S6, and 4E-BP1. Immunoblotting confirmed the RPPA results, i.e., the ability of DQ661 to inhibit autophagy and mTORC1 activity and to induce apoptosis in BRAFV600E, NRASQ61R, and BRAFV600E/NRASQ61R, melanoma cell lines (Fig. 3E). To understand the kinetics of pathway inhibition, immunoblotting revealed that mTORC1 activity was inhibited within 30 minutes of treatment with DQ661, before appreciable accumulation of AVs was observed, as seen by LC3 lipidation (Fig. 3F). Autophagy modulation and dephosphorylation of a number of mTORC1 target substrates was sustained even at the 24-hour time point. Cleavage of caspase-7 did not occur until mTORC1 and autophagy were both significantly inhibited. A kinetic analysis of LMP induction was also performed, revealing LMP does not occur until 2 to 4 hours after treatment with DQ661, suggesting the rapid mTORC1 inhibition associated with DQ661 is independent of LMP (Fig. 3G). Further, Leu-Leu methyl ester hydrobromide (LloMe), a prototypical lysosomal membrane permeabilizing agent, did not inhibit mTORC1 activity at the 30-minute time point when LloMe-induced LMP was clearly evident, demonstrating that DQ661-induced LMP is not responsible for mTOR inhibition (Supplementary Fig. S3B).

To determine whether the anti-mTORC1 activity of DQ661 could be observed with other agents that impair lysosomal deacidification, produce LMP, or inhibit lysosomal enzymes, A375P cells were treated with either AA/serum starvation, asparaginase inhibitor pepstatin A, cysteine protease inhibitor E64, Pepstatin A + E64, acid sphingomyelinase inhibitor siramesine (22), HSP70 inhibitors PES or PET (23), baf, or baf + DQ661 (Fig. 3H). Immunoblotting confirmed DQ661 inhibits mTORC1 activity to a similar level as serum starvation and to a greater extent than any other lysosomal agent tested. PET inhibited S6K phosphorylation, but both PES and PET treatment led to the hyperphosphorylation of 4E-BP1. Baf had minimal effects on phospho-S6K and produced hyperphosphorylation of 4E-BP1. DQ661 had the greatest ability to inhibit autophagy among the entire panel of lysosomal inhibitors, as seen by the greatest accumulation of the LC3II/LC3I ratio. At the doses and time points tested, DQ661 appears to be unique in its ability to inhibit both mTORC1 signaling and autophagy (Fig. 3H). In contrast to these effects observed with DQ661, RPPA data and Western blot analysis demonstrated that neither QC nor DQ660 had a significant impact on mTORC1 signaling at equimolar doses (Supplementary Fig. S3A and S3C).

mTORC1 inhibition upregulates not only autophagy, but also macropinocytosis, a process by which nutrient-limited, Ras-driven cancer cells endocytose, envelop extracellular protein, and release AAs through lysosome-mediated degradation, paradoxically fueling growth of cancer cells (24). We next asked whether or not DQ661 would increase lysosomal catabolism of extracellular proteins because it inhibits mTORC1. We confirmed that DQ661 blocked autophagy and mTOR in G43 mouse pancreatic cancer cells (Supplementary Fig. S3D) derived from the LSL-KrasG12D/+; Pdx1-Cre (KPC) mouse model (25). Neither torin-1 nor DQ661 significantly changed the level of macrophagic uptake of albumin in glutamine-limited G43 cells (Supplementary Fig. S3E). Torin-1 treatment significantly increased lysosomal degradation of externally supplied protein, as displayed by the unquenched fluorescence of DQ-BSA. In contrast, treatment with DQ661 did not produce increased lysosomal degradation of external proteins when G43 cells were grown in similar conditions (Supplementary Fig. S3E). Further, torin-1 significantly increased the proliferation of glutamine-limited cancer cells, confirming that all DQs lacking a centrally methylated nitrogen (DQXX0) significantly increase autophagic flux (Fig. 2D). In contrast, every DQ with central nitrogen methylation (DQXX1) did not demonstrate an increase in LC3II/LC3I ratio in baf-treated cells compared to control (Fig. 2D). This difference in autophagy modulation between unmethylated and methylated DQs was also evidenced by unmethylated DQs producing striking degradation of the autophagy substrates p62 and NBR1, whereas treatment with methylated DQs did not (Fig. 2E). Taken together, these data indicate that DQs without central nitrogen methylation function as autophagy inducers, whereas DQs with central nitrogen methylation modulate autophagy in a more pronounced fashion, acting as autophagy inhibitors. To further confirm that central nitrogen methylation dictates subcellular localization of DQs, lysosomal fractionation was performed in cells treated with either DQ660 or DQ661. Quantification of DQ660 and DQ661 concentrations in subcellular compartments revealed a 3.5-fold difference in the concentration of DQ661 compared with DQ660 in lysosomal fractions (Fig. 2F).
Figure 3. Central nitrogen methylation status dictates DNA damage versus lysosomal membrane permeability. A, Immunofluorescence (IF) microscopy of A375P cells treated as indicated (6 hours, 3 μmol/L) and stained for phospho-H2AX (red; arrows) and DAPI (blue); and scored using ImageJ (mean ± SD). *, P < 0.05. B, IF microscopy of A375P cells treated as in A with the addition of the positive control LloMe (3 hours, 2 mmol/L). IF against galectin-3 is shown. White arrows: galectin-3 puncta, reflecting lysosomal membrane permeabilization. C, Lysosomal subfractionation and immunoblotting in A375P cells treated as indicated (6 hours, 3 μmol/L). WC, whole lysate; L, lysosomal fraction. D, A375P cells were treated with DQ661 (24 hours, 3 μmol/L), and lysate was immunoblotted. MT, mutant; WT/WT, BRAF wild-type and NRAS wild-type. E, A375P cells were treated with DQ661 (0–4 hours; 3 μmol/L), and lysate was subjected to RPPA. Shown are graphs reflecting fold decrease of mTORC1 substrates. F, A375P cells were treated with the addition of the positive control LloMe (3 hours, 2 mmol/L). IF against galectin-3 is shown. Red arrows are indicate galectin-3 puncta. Right, quantitation of percentage of galectin-3 puncta-positive cells. H, A375P cells were treated with DQ661 (6 hours, 3 μmol/L), PBS, Pepstatin A (10 μg/mL), E64 (10 μg/mL), PepAE64, Siramesine (8 μmol/L), PES (10 μg/mL), PET (10 μg/mL), Bafilomycin A1 (100 nmol/L), or Bafilomycin A1 + DQ661. Lysate was immunoblotted against phospho-S6K T389, total S6K, phospho-4E-BP1 S65, total 4E-BP1, LC3B, and Actin. I, A375P cells were treated with a CQ, Lys05; QC, DQ660, and DQ661 (6 hours, 3 μmol/L), and lysate was subjected to RPPA. Shown are graphs reflecting fold decrease of mTORC1 substrates. A–B, A Cy5 secondary antibody was used for pH2AX and galectin-3 to avoid spectral overlap with DQ661. Scale bars, 80 μm in A and 25 μm in B.
G43 cells supplemented with 2% albumin, relative to cells grown in low glutamine with 2% albumin treated with vehicle control (Supplementary Fig. S3F). DQ661 decreased the proliferation of G43 cells to the levels observed by cells cultured in low glutamine in the absence of 2% albumin.

To directly measure the effect of DQ661 on catabolism of extracellular protein by pancreatic cells, we devised an isotope tracer-based method to monitor protein scavenging. This method enables the separate quantification of (i) AAAs added to the medium as monomers and (ii) AAAs derived from degradation of extracellular protein taken up by macropinocytosis. Another cell line derived from a KPC mouse tumor, KRKA-C, which is capable of robust proliferation despite AA deficiency in BSA-supplemented medium, was cultured in the medium containing fully 13C-labeled essential AAAs for 5 doublings, such that intracellular protein and AAAs in these cells were isotopically labeled. These cells were then switched to AA-free medium containing 50 g/L BSA. Under these conditions, cells can substitute AA monomers with scavenged extracellular protein, but because BSA contains unequal levels of AAAs relative to their cellular requirements, serum protein catabolism produces some of these AAAs in excess of these requirements and secretes them into the medium, providing a quantitative readout for protein scavenging rate. Using this assay, we observe that DQ661 significantly inhibits protein scavenging in a dose-dependent fashion (Supplementary Fig. S3G). Altogether, these results demonstrate that DQ661 is capable of blocking not only autophagy but also other lysosomal catabolic programs such as macrophagocytosis.

**PPT1 Is a Molecular Target of DQ661**

Given that LMP did not explain the mTOR inhibition observed with DQ661, we leveraged the increased potency and lysosomal localization of DQ661 to perform an in situ chemical pulldown experiment, in order to find the putative molecular target of DQ661 within the lysosome (Fig. 4A). A photoprobe consisting of the DQ661 warhead attached to benzophenone (as a photoaffinity label), which was in turn covalently linked to desthiobiotin, was synthesized (Fig. 4B; see Methods for chemical strategy). Treatment of A375P cells with the DQ661 photoprobe produced accumulation of LC3II and decreased phosphorylation of mTOR substrates in A375P cells, demonstrating that the photoprobe was cell-permeable, localized to the lysosome, and retained activity (Fig. 4C). Next, A375P cells were treated with the DQ661 photoprobe with or without UV irradiation and with or without competition with an excess of free DQ661 ligand immediately prior to UV irradiation. Cell lysis was followed by pulldown with neutravidin beads and elution with biotin. Eluents were subjected to proteomic analysis (see Methods). Candidate protein targets were identified using the criteria of proteins that had >10-fold intensity ratios of +UV/−UV and −competition/+competition samples (Fig. 4D). Of these 15 proteins, cathepsin D and PPT1 had the highest signal intensity for the +UV sample. Unlike cathepsin D, however, treatment with DQ661 competition completely abrogated the ability of the photolabel probe to pulldown PPT1 as detected by MS. Immunoblotting against PPT1 in a separate experiment using the DQ661 photoprobe and 10x concentration of free DQ661 ligand as competition confirmed that the DQ661 photoprobe specifically bound to and could be UV conjugated to PPT1 in cells (Supplementary Fig. S4A). Cathepsin D enzymatic activity was not significantly impaired in cell lysates from A375P cells treated with vehicle, DQ660, and DQ661 (Fig. 4E). In contrast DQ661, but not DQ660, significantly impaired PPT1 enzymatic activity in cells (Fig. 4F). The inability to inhibit cathepsin D enzymatic activity demonstrates the selectivity of DQ661 for PPT1, further supporting a specific rather than generic mechanism of action for DQ661 in the lysosome. Finally, differential scanning calorimetry (DSC) of recombinant PPT1 protein in the absence or presence of a 4-fold molar excess of DQ661 revealed protein melting temperatures of 67.16°C and 64.60°C, respectively (Fig. 4G). This statistically significant difference of >2.5°C in protein melting temperature, as a function of added DQ661, is consistent with direct DQ661 binding to PPT1.

PPT1 is the only lysosomal enzyme that cleaves thioester bonds from palmitoylated proteins, liberating these proteins from membranes to facilitate proteolysis. An acyl-biotinyl exchange (ABE) assay to measure protein palmitoylation demonstrated that accumulation of palmitoylated proteins occurred as early as 30 minutes and was sustained after 2 hours of treatment with DQ661 (Supplementary Fig. S4B). As proof of principle, the palmitoylated form of the prototypically palmitoylated protein CD44 accumulated after 30 minutes of DQ661 treatment (Fig. 4H). siRNA knockdown of PPT1 phenocopied DQ661 effects, as evidenced by accumulation of lipiddated LC3 and p62, the abrogation of S6 phosphorylation, the inhibition of cellular proliferation, the cleavage of caspase-7, and induction of cancer cell death (Fig. 4I; Supplementary Fig. S4C and S4D). The palmitate mimetic hexaethylene sulfonyl fluoride (HDSF; Supplementary Fig. S4E), a low-potency tool compound that serves as an irreversible inhibitor of PPT1 (26), also concurrently inhibited mTOR and autophagy (Supplementary Fig. S4F). Importantly, cotreatment of melanoma cells with DQ661 and the thioesterase mimetic N-(tert-butyl) hydroxylamine (NtBuHA) partially rescued mTORC1 signaling and autophagy levels (Fig. 4J). siRNA knockdown of PPT1 also partially blunted the ability of DQ661 to inhibit mTORC1 and autophagy (Supplementary Fig. S4G). These results further demonstrate that the biological activity of DQ661 is mediated through inhibition of PPT1-dependent depalmitoylation of palmitoylated proteins.

**DQ661 Ejects mTOR from the Lysosome Membrane, Disrupting mTORC1–RHEB Interactions**

We hypothesized that PPT1 inhibition by DQ661 could lead to mTOR inhibition through an “inside-out” signaling pathway connecting v-ATPase (v-ATPase) and Ragulator interactions. Recently, the regulation of v-ATPase localization by PPT1 was described in neurons (27). The AA-dependent physical interaction between v-ATPase and the Ragulator complex facilitates the lysosomal recruitment of mTORC1 by RAG GTPases (28). This recruitment to the lysosome surface is critical for the mTOR–RHEB interaction, allowing RHEB to phosphorylate and activate mTOR (Fig. 5A). Membrane fractionation of cells treated with DQ661 revealed a significant displacement of multiple components of the v-ATPase from the membrane fraction to the cytoplasmic fraction (Fig. 5B). The physical interaction between subunit V1A (ATP6V1A) and the Ragulator component p18 was also...
Figure 4. PPT1 is a target of DQ661. A, Schematic demonstrating the pulldown strategy. B, Chemical structure of the DQ661-photoprobe. C, A375P cells were treated with the DQ661-photoprobe [24 hours, 0–100 μmol/L], and lysates were immunoblotted. D, Graph depicting the mass spectrometry (MS) analysis of lysate from pulldown with the DQ661-photoprobe. Conditions analyzed were cells that were treated with the DQ661 photoprobe ± UV and ± 10× concentration of DQ661 competition. E, Cathepsin activity in A375P cells following treatment with DQ660 or DQ661 (3 hours, 3 μmol/L). F, PPT1 enzymatic activity in A375P cells following treatment with DQ660 or DQ661 (3 hours, 3 μmol/L). *, P < 0.05. G, In vitro binding of DQ661 to PPT1. Differential scanning calorimetry of 1 mg/mL PPT1 (29.4 μmol/L) in the absence (black) or presence (green) of DQ661 (100 μmol/L). Tm, melting temperature. H, A375P cells were treated with DQ660 (0–240 minutes, 3 μmol/L). CD44 palmitoylation measured using the ABE assay increases with DQ661 treatment compared with control. Samples not treated with hydroxylamine (−HAM) serve as a negative control. I, A375P cells were treated with 25 nmol/L PPT1 siRNA or nontargeting (siNT). Cells were transfected overnight in the absence of serum. Upon serum restimulation, cells were collected 3 or 6 hours thereafter, and lysate was immunoblotted. J, A375P cells were treated with DQ661 (1 hours, 3 μmol/L) in the presence or absence of NTBHA (2 mmol/L). Lysate was immunoblotted.
disrupted by DQ661, as seen by proximity ligation assay (PLA; Fig. 5C). Immunofluorescence microscopy revealed that p18 colocalization with lysosomes was not affected by DQ661 (Fig. 5D). In contrast, DQ661 treatment significantly displaced RAGC (Fig. 5E) and mTORC1 (Fig. 5F) from the lysosomal surface. Immunoblotting of lysosome fractions taken from A375P cells treated with vehicle or DQ661 further corroborated the DQ661-induced displacement of RAGC and mTOR from the lysosome and into the extralysosome fraction (Fig. 5G). In addition to displacement, DQ661 also breaks the physical interaction between RAG GTPases and mTOR, as seen by PLA (Fig. 5H) and immunoprecipitation of RAGB in cells expressing FLAG-RAGB, followed by immunoblotting for mTOR (Fig. 5I). In contrast, neither rapamycin nor torin-1 could displace mTOR from the lysosome (Supplementary Fig. S5A). As RHEB is the master activator of mTORC1, we next interrogated whether DQ661, existing lysosome-targeting agents, or existing mTORC1 inhibitors could affect the physical interaction between mTORC1 and RHEB. Neither rapamycin nor torin-1 was able to disrupt the physical interaction of mTOR and RHEB, as seen by PLA (Fig. 5J). Similarly, although neither baf nor the LMP inducer siramesine disrupted the mTOR-RHEB interaction, which is critical for mTORC1 growth signaling, this interaction was completely disrupted by DQ661 (Fig. 5I). The unique ability of DQ661 to disrupt the mTOR-RHEB interaction is therefore not shared by allosteric or catalytic inhibitors of mTORC1 nor by other prototypical lysosomally directed cationic amphipathic drugs (CAD). Next, incubation of cells with O-propargyl-puromycin followed by click chemistry–enabled attachment of a fluorophore was used to compare the effects of mTOR and lysosomal inhibitors on de novo protein translation (29). Catalytic inhibition of mTORC1 decreased protein translation to a significantly greater degree than allosteric inhibition of mTORC1. Meanwhile, lysosomal inhibition alone with baf had no effect on protein translation. In contrast, concurrent inhibition of mTORC1 and lysosomal catabolism with DQ661 was the only approach that produced near-complete inhibition of protein translation (Supplementary Fig. S5B).

We observed striking differences in the antiproliferative activity of catalytic/allosteric mTOR inhibitors compared with DQ661. Although torin-1 displays significantly greater antiproliferative activity relative to rapamycin, the growth impairment reaches a plateau at 1 μmol/L whereby a residual population of cancer cells persists despite increasing dosages...
In Vivo Lysosomal Inhibition and Antitumor Activity of DQ661 in a Melanoma Xenograft Model

To determine whether DQ661 possesses in vivo activity, 1205Lu (BRAFV600E-mutant, HCQ-resistant; ref. 11) melanoma xenografts were established on the flanks of NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. After 1 to 2 weeks, tumors were palpable and mice were treated intraperitoneally (i.p.) with vehicle control (water), QN, DQ660, or DQ661 (8 mg/kg; Fig. 6A). QN had no significant effect on tumor growth compared with vehicle control. In contrast, DQ660 treatment resulted in a modest growth impairment of tumors compared with control or QN-treated tumors. DQ661 inhibited tumor growth to a significantly greater extent than DQ660, despite differing in structure by only one methyl group. Further, DQ661 was the only compound to produce a nearly 0% growth rate (Fig. 6B). Immunohistochemistry of tumor slides revealed the presence of H2AX only in the DQ660-treated mice (Fig. 6C). Electron microscopy demonstrated DQ661 alone produced visible accumulation of AVs in tumor cells (Fig. 6D). At the 8 mg/kg dose, however, DQ661 caused weight loss greater than 10%, and by day 7 all mice were euthanized due to lethargy and bowel distension (Supplementary Fig. S6A). Histologic analysis of the intestines demonstrated Paneth cell dysfunction only in DQ661-treated mice, similar to that observed in mice treated with the highest doses of Lys05 (ref. 11; Supplementary Fig. S6B). Therefore, another 1205Lu xenograft experiment was performed in which mice were treated with vehicle control (water) or DQ661 4 mg/kg i.p. daily for a period of 14 days. Treatment with DQ661 resulted in a significant reduction in tumor volume compared with control mice without significantly affecting mouse weight (Fig. 6E; Supplementary Fig. S6C). Mice treated with 4 mg/kg DQ661 showed significantly slower rates of tumor growth (Fig. 6F). Evidence of in vivo mTORC1 and autophagy inhibition was observed through immunoblotting of lysate from treated tumors (Fig. 6G). To ensure the effects of DQ661 were not due to a predisposition of 1205Lu cells to mTOR or autophagy inhibition, genetic knockdown with siRNA against ATG5 or mTOR was performed and no significant impact on viability was
Figure 6. DQ661 has significant single-agent in vivo activity in melanoma xenograft model. A, 1205Lu cells were injected subcutaneously in the flanks of NSG mice (2 × 10^6 cells/mouse) and grown until tumors were palpable. Mice (n = 8/arm) were treated with vehicle control (water, i.p.), quinacrine (8 mg/kg, i.p.), DQ660 (8 mg/kg, i.p.), or DQ661 (8 mg/kg, i.p.). Treatments were given as shown by the black arrows (2 days on, 2 days off). Mean ± SEM is presented; *, P < 0.05. A linear mixed-effect model was used to test the difference of the tumor growth trends among treatment groups. B, Mean ± SD tumor growth rate. C, Tumor tissues stained for phospho-H2AX Ser139. Scale bar, 40 μmol/L. D, Representative electron micrographs of tumors harvested from mice after 2 days of treatment with each agent. Arrows: autophagic vesicles. Scale bars, 730 nm. E, 1205Lu cells were injected subcutaneously in the flanks of NSG mice (2 × 10^6 cells/mouse) and grown until tumors were palpable (1–2 weeks). Mice (n = 8/arm) were treated with water (i.p.) or DQ661 (4 mg/kg, i.p.). Black arrows: treatment schedule (2 days on, 2 days off). Mean ± SEM are presented for daily tumor volumes; *, P < 0.05. F, Average tumor growth rate. G, Protein lysate from mouse tumors at the end of the experiment was immunoblotted as indicated. H, In vivo PLA in mice treated with vehicle control or 4 mg/kg DQ661. Red fluorescence indicates mTOR–RHEB interaction; blue, DAPI staining of nuclei.
DQ661 Improves Survival in a Colon Cancer Model and Has Antitumor Activity in an Immunotherapy-Resistant Pancreatic Syngeneic Model

Autophagy serves a critical role in the survival of colorectal cancer cells under hypoxic conditions as well as a resistance mechanism to antiangiogenic therapy (30). A recent phase I clinical trial of FOLFOX, bevacizumab, and HCQ in colon cancer showed significant activity (Dr. Mark O’Hara, personal communication), leading to additional clinical trials in colon cancer involving lysosomal autophagy inhibition. To determine whether DQ661 possessed activity in a colorectal mouse model, HT-29 cells were subcutaneously injected into the flanks of NSG mice. DQ661 treatment significantly decreased tumor volumes compared with control (Fig. 7A). Treatment with DQ661 was well tolerated and significantly increased the survival of mice (Supplementary Fig. S7A and Fig. 7B). Tumor growth rate was significantly impaired with DQ661 treatment (Fig. 7C).

Pancreatic ductal adenocarcinoma (PDA) requires high levels of autophagy–lysosome function to maintain metabolism and resist therapy (12, 31). The combination of gemcitabine and HCQ has been shown to be synergistic in an orthotopic model of KPC pancreas cancer. Further, clinical translation of this finding was performed in a neoadjuvant trial of gemcitabine and HCQ in locally advanced pancreas cancer with encouraging results (32). We next determined whether DQ661 has activity against treatment-naïve, as well as immunotherapy/radiation-resistant, pancreas cancer cells. Two mouse cancer cell lines were derived from the KPC genetically engineered mouse model (GEMM): the PDA.4662 cell line (parental) and the G43 cell line. The latter was generated from a PDA.4662 tumor that grew in the face of CTLA4 blockade combined with radiation therapy (25). The immunotherapy-resistant G43 cells were found to be resistant to gemcitabine, relative to the parental treatment-naïve PDA.4662 cells (Fig. 7D). Gemcitabine was found to concurrently increase mTORC1 activity and autophagy as resistance mechanisms (Fig. 7E). To determine whether DQ661 would potentiate the effects of gemcitabine, G43 cells were treated with gemcitabine in the presence or absence of DQ661 in long-term colony formation assays. DQ661 significantly enhanced the antitumor activity of gemcitabine against G43 cells (Fig. 7F). To determine whether DQ661 would significantly enhance the in vivo activity of gemcitabine, G43 cells were implanted in the flanks of syngeneic mice treated with vehicle control or with a single dose of gemcitabine (120 mg/kg), combined with repeated doses of either additional vehicle or DQ661 (4 mg/kg; Fig. 7G). Both gemcitabine alone and DQ661 alone significantly impaired tumor growth. However, compared with DQ661 alone, or gemcitabine alone, the combination of DQ661 and gemcitabine produced a significantly lower tumor growth rate and was well tolerated (Fig. 7H; Supplementary Fig. S7B). These results demonstrate DQ661 has activity in colorectal xenografts and immunocompetent mouse models of pancreatic cancer and has activity in cancers that have developed resistance to immunotherapy/radiation therapy.

DISCUSSION

Here, we perform the first-ever anticancer screen of novel dimers of antimalarial compounds. We have discovered an unprecedented correlation between chemical structure and subcellular localization. This provides a chemical strategy to more specifically target compounds to the lysosome. The potency of the optimized compound was leveraged to identify the protein target of this compound, PPT1. PPT1 inhibition results not only in autophagy inhibition but also in mTOR inhibition, through the disruption of mTOR recruitment to the lysosome under both nutrient-limiting and nutrient-replete conditions.

DQ661 was developed by systematic modification of linker length, aromatic heterocycle, and central nitrogen methylation of our previously described compound Lys05. Extending the triamine linker of Lys05 with the spermidine linker led to DC340, which demonstrated significantly increased anticancer activity. Replacing the CQ rings of DC340 with MQ, PQ, or QN revealed that the strategy of dimerization significantly improved the anticancer efficacy with all antimalarial heterocycles tested. Our results show that the historically oldest antimalarial substance (QN) has the greatest anticancer activity on dimerization. The effectiveness of these dimeric compounds in cancer models suggests that they should be investigated in other disorders where CQ has shown efficacy, including rheumatoid arthritis (33), malaria (34), prion disease (35), and viral disorders (36).

Extension of our DQ library using longer linkers established an optimal distance between the two QN moieties of 9 to 15 atoms. Furthermore, linker length correlated directly with the ability of DQs to induce apoptosis and modulate autophagy. The limited potency of DQ221, which has the same linker length as Lys05, could be attributed to the increased steric demands of the tricyclic acridine heterocycle in QN compared with the bicyclic quinoline moiety of CQ. QN possesses both lysosomal and extralysosomal functions in the cell (37). Methylation of the central nitrogen plays a critical steering role in localizing the DQs to the lysosome in a pH-dependent manner, triggering striking levels of LMP. In contrast, DQs lacking central nitrogen methylation do not induce LMP and instead produce DNA damage. Linker length correlated directly with the level of induced DNA damage, as well as with the induction of autophagic flux among the unmethylated DQs. With the methylated DQs, linker length directly correlated with the induction of LMP and with autophagy inhibition. The clear dependence of anticancer activity on linker length strongly supported a specific interaction of DQ661 with a protein target, and not with a general deacidification mechanism, as suggested for the monomeric compounds and other previously described CADs. Furthermore, the remarkable selectivity in localization as a function of nitrogen methylation cannot be attributed to a simple difference in pKa. The differences observed between the unmethylated and methylated amines could be attributed to Schiff base formation, which is available only to the unmethylated secondary amines.

The DQs are CADs, which have been reported to possess anticancer activity, neutralize lysosomal pH, induce lysosomal leakage, inhibit autophagy, and blunt drug resistance (38). However, CADs often have multiple effects within the cell and can, in some cases, induce or inhibit autophagy. CADs such as amiodarone, fluoxetine, imipramine, and
Figure 7. DQ661 improves survival in colon cancer model and potentiates activity of gemcitabine in KPC pancreatic cancer syngeneic model. A, HT-29 cells were injected s.c. into the flanks of NSG mice and grown until tumors were palpable. Mice (n = 8/arm) were treated with water or DQ661 (4 mg/kg) i.p. DQ661-treated mice were treated 2 days on, 2 days off. Mean ± SEM is presented. B, Survival curve for A displaying the time it took for mice to reach death (defined as time when tumor volume exceeded 1000 mm³). C, A linear mixed-effect model was used to test the difference of the tumor growth trends among treatment groups from A. Mean ± SEM tumor growth rate. D, MTT assay of KPC cell lines 4662 and G43 treated with gemcitabine (72 hours, 3–30 nmol/L). *, P < 0.05. E, G43 cells were treated with gemcitabine (24 hours, 10 nmol/L) and lysate was immunoblotted. F, G43 cells were treated chronically for 2 weeks with gemcitabine (3–30 nmol/L) in the presence or absence of DQ661 in colony formation assays. Cells were stained with crystal violet and imaged. G, G43 cells were injected subcutaneously into the flanks of C57BL/6 mice (2 × 10⁶ cells/mouse). Once palpable, mice (n = 8 mice/treatment arm) were treated with vehicle (PBS), gemcitabine (120 mg/kg, i.p.), DQ661 (4 mg/kg, i.p., 2 days on, 3 days off), or a combination of gemcitabine and DQ661. H, A linear mixed-effect model was used to test the difference of the tumor growth trends among treatment groups from G. Mean ± SEM tumor growth rate.

amitriptyline all have been shown to induce autophagy and have no effect on mTOR signaling (39–42). Our study demonstrates that incorporating a specific structural motif (central nitrogen methylation of a triamine linker) in a dimeric compound can enhance lysosomal localization and increase the specificity of CADs as antilyosomal agents. Because the unmethylated compounds tested (DQ330, DQ440, DQ550, and DQ660) are all CADs, this was the most stringent demonstration that not all CADs share these biological properties, and that lysosomal localization and specificity with respect to mechanism of action can be optimized through chemical modification.

DQ661 is the first example of a lysosome-targeted agent that can mechanistically inhibit mTORC1 in the context of
nutrient-rich conditions via disruption of mTOR. One previous study demonstrated that CADs as well as lysosome deacidifying agents possess anti-mTORC1 activity specifically due to lysosome impairment (43). The compounds investigated in that study included siramesine, concanamycin A, bafilomycin A, and ammonium chloride. Siramesine inhibits lysosome function by inhibiting acid sphingomyelinase (ASM; ref. 22). However, no connection between mTORC1 signaling and ASM inhibition was made in this previous report, and our data indicate that siramesine in nutrient-replete conditions cannot break the mTOR–RHEB interaction in the same manner as DQ661. Decadification of the lysosome with concanamycin A, baf, and NH₄Cl results in robust inhibition of mTORC1 only in the context of limited nutrients when cells are heavily reliant on lysosomal recycling for survival. Studies from the laboratories of Thompson and Sabatini have confirmed that, in the setting of AA-replete media, agents that target lysosomal acidification and/or the vacuolar ATPase fail to inhibit mTORC1 (24, 28, 44). DQ661 is the first example of a lysosomally targeted agent that can impair mTORC1 signaling in the presence of AAs.

The v-ATPase, known to be responsible for the acidification of the lysosomal lumen, also aids in the lysosomal recruitment of mTORC1 through AA-dependent physical interactions with the Ragulator complex (28). Our mechanistic studies demonstrate that the inhibition of PPT1 initiates a cascade, beginning with the lysosomal displacement of v-ATPase subunits, which disrupts the function of Ragulator and RAG GTPase machinery, impairing lysosomal recruitment of mTORC1 and preventing RHEB-dependent activation of mTOR. Further study is warranted to determine the roles of the mTOR recruitment machinery (regulator–RAG complex), the vacuolar ATPase, and the TFE3/TFEB family of transcriptional regulators of lysosomal biogenesis in DQ661 activity and resistance.

Targeting mTORC1, which is constitutively activated in >70% of all cancers, with small-molecule inhibitors remains a challenge due to limited efficacy and the onset of resistance and toxicity (7, 45). Acquired resistance mechanisms to existing mTOR inhibitors include mTOR mutations. A third-generation mTOR inhibitor, RapaLink-1, can overcome these acquired resistance mechanisms (46). However, lysosome-dependent catabolic programs such as autophagy and macroautophagy represent mutation-independent adaptive resistance mechanisms to mTOR inhibition (12, 24). By targeting PPT1, DQ661 concurrently inactivates mTORC1 function and overcomes these adaptive resistance mechanisms.

Finally, this work has identified PPT1 as an important new target in cancer. Germline mutations in PPT1 can result in infantile neuronal ceroid lipofuscinosis (INCL or CLN1), a fatal disease in which children develop normally in utero, but develop neuronal degeneration in early childhood. Although lipofuscinosis has many of the hallmarks of defective autophagy, to date the characterization of autophagic flux and mTOR signaling in INCL cells has not been reported. The fact that PPT1 modulates protein palmitoylation, and DQ661 has now been shown to impair this enzymatic function, is important, because many of the critical regulators of autophagy and mTOR signaling are predicted to be palmitoylated proteins (47). Studies investigating the link between protein palmitoylation and lysosomally controlled signaling are ongoing.
**Macropinocytosis Experiments**

KPC pancreatic cancer cells were plated (100,000 cells/well) upon glass cover slips and cultured in DMEM media containing 10% dialyzed FBS (Gemini Bio-Products), 0.2 mmol/L glutamine (0.2Q), and 2% albumin (0.2Q + Alb). To measure macropinocytic uptake and lysosomal Catalysis of ingested protein, cells were either fed BSA-647 (Thermo Fisher Scientific A34785) or DQ-BSA (Thermo Fisher Scientific D12051) for 3 hours. Cells were subsequently washed 4 times with cold PBS, fixed with 4% paraformaldehyde. KRPC cells were cultured for 5 doublings in DMEM containing fully 13C-labeled essential histidine, lysine, phenylalanine, threonine, tyrosine, and valine, such that intracellular protein and AAs in the resulting cells were mostly labeled. After one PBS wash, these cells were switched to AA-free medium supplemented with 5% unlabeled BSA. Excretion of unlabeled AAs into the medium was monitored after 8 hours. AAs were extracted from the medium and subjected to LC/MS analysis as described previously (51).

**Fluorimetry**

Small-molecule fluorescent properties were observed on a Tecan M1000 fluorescent plate reader. UV-Vis spectroscopy was collected in triplicate, and 420 nm was found to be the average most redshifted absorption across the library of DQs. Emission spectra were collected at 1 μmol/L, which was confirmed to be in the linear range of fluorescence. Emission data were processed with a five-point floating average to reduce instrument noise. The data were then normalized to the fluorescence maximum of the library, DQ661.

**Measurement of Drug Concentration in Subcellular Fractions**

The fluorescence emission intensity (ex. 424, em. 495) of each inhibitor, DQ660 or DQ661, was measured at multiple concentrations in PBS. From these data, a separate linear calibration curve was made for each drug. Lysosomal and whole-cell fractions were diluted 1:100 in PBS, and nuclear and cytosolic fractions were diluted 1:10 in PBS. The fluorescence emission intensity (ex. 424, em. 495) of each of these diluted samples was measured and converted into a concentration by utilizing each inhibitor’s respective calibration curve and corrected for dilution. The resulting drug concentration values of each cellular extract were then normalized for protein content and compared. Error is reported as the standard deviation of each fluorescence measurement.

**Protein Translation**

Cells were treated as described, and nascent protein synthesis was detected utilizing immunofluorescence microscopy according to the manufacturer’s instructions (Click-iT Plus OPP Protein Synthesis Assay Kit C10458).

**Membrane Fractionation**

Membranes were fractionated out according to the manufacturer’s instructions (Subcellular Protein Fractionation Kit, Thermo, #78840).

**Proteomics**

DQ661 (DQ) pulldowns from cell lysates were separated for 0.5 cm on 10% NuPage gels (Life Technologies, NP0360), stained using coomassie Coomassie (Life Technologies, LC6025), and the entire stained region was digested with trypsin, prior to LC/MS-MS analysis using nanoACQUITY UPLC (Waters) in line with a QExactive Plus mass spectrometer (Thermo Fisher Scientific), using conditions similar to those previously described (52). Proteins were identified using MaxQuant version 1.5.2.8 (http://www.maxquant.org; ref. 53) with the UniProt human sequence database (August 29, 2016; 176,545 sequences, 61,529,373 residues) and an appended expected contaminants database. Protein and peptide false discovery rates were set at 1%, and other critical MaxQuant parameters were as described recently (52). Proteins identified by at least two unique peptides were further analyzed using label-free quantitation values to determine the ratios of protein intensities in DQ + UV relative to the two controls (DQ No UV and DQ + Competitor + UV) for each protein. If DQ + UV protein intensity was zero, the ratio was reported as not detected (N.D.), whereas if the protein intensity in a control was zero, the ratio was reported as an arbitrary value of 1,000 to avoid division by zero. Proteins showing a ratio of greater than 10-fold were reported as potential interactors.

**Purification of PPT1**

Full-length PPT1 plasmid was obtained from Addgene (Plasmid #25205). Sf9 cells grown at a density of 3 × 10⁶ cells per milliliter of media were infected with 10 mL/L of P2 viral stock. Cell culture medium was collected following 96 hours of incubation at 27°C. Final cell viability was 48%. The cultured medium was centrifuged at 4,000 × g for 30 minutes. Secreted PPT1 protein was collected using ammonium sulfate precipitation at 60% saturation. Media were stirred with ammonium sulfate for 1 hour at 4°C, and precipitated protein was collected by filtration. The precipitate was resuspended in resuspension buffer (20 mmol/L HEPES pH 7, 150 mmol/L NaCl) and purified on Ni-NTA resin. Following washing (resuspension buffer plus 20 mmol/L imidazole) and elution (resuspension buffer plus 300 mmol/L imidazole), the protein was further purified on an s200 gel filtration column (20 mmol/L Bis-Tris pH 6, 150 mmol/L NaCl).

**DSC**

Protein was concentrated to 1 mg/mL (29.4 μmol/L) and dialyzed for 18 hours into 20 mmol/L NaOAc pH 5, 50 mmol/L NaCl. DSC experiments used a MicroCal VP-Capillary DSC with and without the addition of 100 μmol/L DQ661. Scans were run from 10°C to 90°C using a scan rate of 60°C/hour and a filter period of 10 seconds.

**PPT1 and Cathepsin D Enzyme Assays**

PPT1 enzymatic activity was measured as previously described (54). Cathepsin D enzyme assay was performed according to the manufacturer’s instructions (Abcam ab65302).

**ABE Assay**

The protocol is adapted from ref. 55. Cells were harvested in lysis buffer (50 mmol/L HEPES pH 7.4, 1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L N-ethyl-maleimide (NEM), 1 μg/mL leupeptin, 1 μg/mL aprotinin, 2 μg/mL peptatin A. Lysates were clarified by centrifugation at 15,000 RPM for 10 minutes. The lysate was methanol/chloroform (m/c) precipitated, and the dried pellet was resuspended in 40 μL 4% SDS buffer + 50 mmol/L NEM and incubated at room temperature for 1 hour. The samples were m/c precipitated twice and then resuspended in 80 μL 4% SDS buffer. The samples were split in half, and 160 μL of hydroxylamine buffer (0.7 mol/L hydroxylamine pH 7.4, 50 mmol/L HEPES pH 7.4, 0.2% Triton X-100, 150 mmol/L NaCl, 5 mol/L EDTA) was added to one half of the sample, and control 0.2% Triton X-100 buffer (50 mmol/L HEPES pH 7.4, 0.2% Triton X-100, 150 mmol/L NaCl, 5 mol/L EDTA) was added to the remaining sample and incubated at room temperature for 1 hour. The samples were m/c precipitated and resuspended in 40 μL 4% SDS buffer containing 10 μmol/L Biotin–HPDP. Then, 160 μL of 0.2% Triton X-100 buffer + 10 μmol/L Biotin–HPDP was added and incubated at room temperature for 1 hour. The samples were m/c precipitated and resuspended in 20 μL of 4% SDS buffer followed by addition of 180 μL of 1% Triton X-100 buffer (20 μL removed for analysis as “input”). The sample was further diluted with 600 μL of 1% Triton X-100 buffer, and 30 μL of streptavidin agrose beads were added to the samples and incubated overnight at 4°C rotating. The samples were washed in 1% Triton-X100 buffer and analyzed by SDS-PAGE.
In Vivo Mouse Studies

NSG mice (The Jackson Laboratory) were used for xenograft studies. Tumor generation, tumor measurement, and tumor harvesting were performed as previously described (48). For the pancreatic cancer model, 2 × 10^6 G43 cells were subcutaneously injected with an equal volume of Matrigel (BD) over the right flank of C57BL/6 mice (The Jackson Laboratory) and tumors were treated when they became palpable. Tumors were measured using electronic calipers, and volume was calculated as L x W^2 x 0.5. Tumor harvesting procedure and immunohistochemistry and EM were performed as previously described (48). All animal experiments were performed in accordance with the protocols approved by the Institute of Animal Care and Use Committee of the University of Pennsylvania.

Statistical Analysis

Statistical significance was determined by using Student unpaired, two-tailed t test. For in vivo longitudinal growth data, linear mixed-effect models were used to test the difference of the tumor growth trends among treatment groups. A P value less than 0.05 was considered as significantly different from the null hypothesis.

Disclosure of Potential Conflicts of Interest

G.B. Mills reports receiving commercial research grants from Adelson Medical Research Foundation, AstraZeneca, Critical Outcome Technologies, Komen Research Foundation, NanoString, Breast Cancer Research Foundation, Karus, Illumina, Takeda/Millennium Pharmaceuticals, and Pfizer; has received honoraria from the speakers bureaus of Symphogen, MedImmune, AstraZeneca, ISIS Pharmaceuticals, Lilly, Novartis, Immunomet, Allostery, Tarveda, and Pfizer; has ownership interest (including patents) in Catena Pharmaceuticals, PTV Ventures, Spindletop Ventures, Myriad Genetics, and Immunomet; and is a consultant/advisory board member for Adventist Health, AstraZeneca, Provista Diagnostics, Signalchem Lifesciences, Symphogen, Lilly, Novartis, Tarveda, Tau Therapeutics, Allostery, Catena Pharmaceuticals, Critical Outcome Technologies, ISIS Pharmaceuticals, Immunomet, Takeda/Millennium Pharmaceuticals, MedImmune, and Precision Medicine. J.D. Winkler has ownership interest in a patent licensed to Presage and two other unlicensed patents. R.K. Amaravadi has ownership interest (including patents) in Lys05, DQ661, and derivatives, and is a consultant/advisory board member for ISIS Pharmaceuticals, ImmunoMet, and Takeda/Millennium Pharmaceuticals; and is a consultant/advisory board member for Presage, Sprint Biosciences, and Immunaccell. No patents) in Lys05, DQ661, and derivatives, and is a consultant/advisory board member for Presage, Sprint Biosciences, and Immunaccell. No

Acknowledgments

We would like to thank Elise Arbeille for assistance with imaging and Andy J. Minn and Robert H. Vonderheide for providing the G43 cell line. We acknowledge the assistance of the Wistar Institute Proteomics and Metabolomics Core.

Grant Support

This work was entirely supported by NIH grants R01CA169134, P01 CA114046, P30 CA016520, SPORE P50 CA174523, 1R01CA198015, CA016672, and P30CA010815.

REFERENCES

4. Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1 activity. eLife 2016;5.
Dimeric Quinacridines Identify New Lysosomal Target in Cancer


A Unified Approach to Targeting the Lysosome's Degradative and Growth Signaling Roles

Vito W. Rebecca, Michael C. Nicastri, Noel McLaughlin, et al.

Cancer Discov  Published OnlineFirst September 12, 2017.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-17-0741

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2017/09/12/2159-8290.CD-17-0741.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.