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Lys05

A new lysosomal autophagy inhibitor

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Lys05 is a previously undescribed dimeric chloroquine which more potently accumulates in the lysosome and blocks autophagy compared with HCQ. Lys05 produced more potent antitumor activity as a single agent both in vitro and in vivo in multiple human cancer cell lines and xenograft models compared with HCQ. Initial structure-activity relationship studies demonstrated that the increased activity associated with Lys05 was due to the bivalent aminoquinoline rings, C7-Chlorine, and a short triamine linker. While lower doses of Lys05 were well tolerated and associated with antitumor activity, at the highest dose tested, Lys05 produced Paneth cell dysfunction and intestinal toxicity, similar to what can be observed in mice and humans with genetic defects in the autophagy gene ATG16L1. Lys05 is therefore a new lysosomal autophagy inhibitor that has potential to be developed further into a drug for cancer and other medical applications.

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Currently, the lysosomotropic chloroquine (CQ) derivatives are one of the only classes of compounds that are both available for clinical trials and associated with distal inhibition of autophagy. Preclinical evidence in mouse models demonstrating that autophagy inhibition with CQ derivatives can augment the efficacy of multiple anticancer agents has led to a number of clinical trials in advanced cancer patients involving hydroxychloroquine (HCQ). A major concern with HCQ is that high micromolar concentrations, which are not being consistently achieved in patients, are required to block autophagy in vitro. While there is some evidence of autophagy blockade in clinical samples, the magnitude of lysosomal dysfunction achievable with HCQ must be improved upon to maximally block functional autophagy and enhance the efficacy of targeted therapies or chemotherapies. Therefore, more potent drug-like autophagy inhibitors are needed. While efforts are underway to target upstream components of autophagy with potent small molecule inhibitors, less attention has been focused on developing better lysosomal autophagy inhibitors. There is increasing appreciation for the redundancy of upstream autophagy components and the complex interplay between macroautophagy, noncanonical macroautophagy, chaperone-mediated autophagy and other components of endovesicular trafficking that contribute to clearance of damaged organelles and recycled nutrients. However, the functional lysosome retains its role as a critical component of bulk degradative pathways, and therefore we chose to focus our efforts on designing, synthesizing and testing novel CQ derivatives that have drug-like properties.

One principle of medicinal chemistry, which demonstrated promise in efforts to improve the potency of CQ as an antimalarial, is the principle of multivalency. CQ and most CQ derivatives in clinical use are monomeric aminoquinolines. Since dimeric CQ derivatives have not been investigated for their effects on autophagy and as anticancer therapeutics, we designed Lys01, a dimeric form of CQ, with the spacer N, N-bis(2-aminoethyl)-methylamine as the connector between

two CQ moieties. We then designed three derivatives of Lys01 that would serve as a preliminary test of the significance of dimerization, the C-7 chlorine, and the linker length for activity. Initial studies demonstrated that all three factors, dimerization, C-7 chlorine, and the linker length contribute to the enhanced activity in autophagy and cytotoxicity assays observed with Lys01. Lys01 treatment of LN229 and LN229 GFP-LC3 glioma cells produce a 10-fold more potent blockade of autophagy compared with CQ or HCQ as evidenced by LC3-II/LC3-I ratio on immunoblotting and the accumulation of large confluent GFP-LC3 puncta using fluorescence microscopy. Electron microscopy confirmed a massive difference in both size and number of autophagic vesicles that accumulate in cells treated with 10 μM Lys01 compared with 10 μM HCQ. A bafilomycin A_1 clamp experiment confirmed Lys01 is an autophagy inhibitor and not an autophagy inducer. The functional consequence of this more potent autophagy inhibition is that Lys01 treatment produces a 3- to 10-fold lower IC_{50} in multiple human cancer cell lines using the 72 h MTT assay compared with CQ or other Lys01 derivatives tested, with more significant differences between Lys01- and HCQ-associated IC_{50}s being observed in cell lines that are highly resistant to HCQ. To conduct in vivo studies we synthesized the water soluble salt of Lys01, Lys05. In two melanoma xenograft models and a colon cancer xenograft model, intermittent high dose Lys05 or chronic daily dosing of Lys05 at lower doses produces significant early blockade of autophagy in vivo, and has single-agent antitumor activity at doses as low as 10 mg/kg i.p. daily. In contrast, single-agent high dose HCQ treatment administered intermittently does not produce clear evidence of autophagy inhibition at early time points, and is associated with tumor growth compared with control in one model. To better understand these findings, the lysosomal drug accumulation and functional deacidification of lysosomes in Lys05 and HCQ treated cells was compared. Compared with HCQ, Lys05 more potently accumulates within and deacidifies the lysosome of both cells and tumors, resulting in more sustained inhibition of autophagy and tumor growth. While even 100 μM HCQ cannot completely deacidify the endovesicular compartment in cancer cells, complete deacidification is observed with 50 μM Lys05 as evidenced by acridine orange aggregation. Finally, at the highest dose administered of Lys05 (80 mg/kg i.p.), mice develop Paneth cell dysfunction associated with loss of lysozyme production, and bowel pseudo-obstruction. Importantly, chronic daily dosing is well tolerated and associated with antitumor activity in mice treated with lower doses of Lys05. The intestinal toxicity associated with high dose Lys05 phenocopies mice and humans with defective autophagy due to inactivating *ATG16L1* polymorphisms. Preliminary evidence available from high dose HCQ trials also indicates patients experience low grade nausea and constipation.

These results provide in vivo evidence that Lys05 is a more potent autophagy inhibitor than existing chloroquines with single-agent antitumor activity. Additional Lys01 derivatives are being tested with the goal of identifying drug-like compounds with nanomolar autophagy inhibition and cytotoxicity profiles. Given that dimerization produces a 10-fold and not a 2-fold change in activity, mass spectrometry pulldown studies are underway to determine if there is a specific molecular target within the lysosome for Lys05 and CQ derivatives, for which dimerization imparts a cooperative advantage. Single-agent studies in transgenic mouse models of cancer, and combination studies with other anticancer drugs are also underway. Additional indications for Lys05 are being explored, including its role as a potential new antimalarial compound.