Supporting Information

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SI Materials and Methods

Synthesis of Compound 3 (Lys01). A round-bottom flask was charged with 4-bromo-7-chloroquinoline (compound **4;** 734 mg, 3.0 mmol), $Pd(OAc)_2$ (23 mg, 0.1 mmol), 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP) (125 mg, 0.2 mmol), K_3PO_4 (1.06 g, 5.0 mmol), and triamine (compound **5**; 117 mg, 1.0 mmol). Dioxane (10 mL) was introduced through the septum. The resulting suspension was stirred under argon at 90 °C for 18 h and cooled. The mixture was adsorbed onto silica gel and purified by flash chromatography (CH_2Cl_2/MeOH: 90/9/1) to afford compound **3** (387 mg, 88%) as a yellow solid. Melting point (mp): 199–200 °C; R_f = 0.28 (silica gel, CH_2Cl_2/MeOH/NH_4OH: 90/9/1): ^{1}H NMR (500 MHz, CDCl3: δ 8.53 (d, J = 5.5 Hz, 2H), 7.94 (d, J = 2.0 Hz, 2H), 7.41 [doublet (d), J = 9.0 Hz, 2H], 6.98 [doublet of doublets (dd), J = 9.0, 2.0 Hz, 2H], 6.39 (d, J = 5.0 Hz, 2H), 5.44 [singlet (s), 2H]. 3.42 [quartet (q), J = 5.0 Hz, 4H], 2.90 [triplet (t), J = 6.0 Hz, 2H], 2.46 (s, 9H). ^{13}C NMR (125 MHz, CDCl3): δ 152.1, 149.5, 149.1, 135.1, 128.9, 125.5, 120.6, 117.1, 99.3, 55.5, 42.4, 40.3. Fourier transform infrared (FTIR; thin film, cm^{-1}): 3215, 2917, 1609, 1579, 1449. High-resolution mass spectra–electrospray ionization (HRMS-ESI; m/z): calculated for $C_{23}H_{24}N_5Cl_2$ $[M + H]^+$: 440.1409, found: 440.1406.

Synthesis of Compound 9 (Lys03). A round-bottom flask was charged with 4-bromo-7-methoxyquinoline **8** (40 mg, 0.2 mmol), $Pd(OAc)_2$ (2 mg, 0.01 mmol), BINAP (12 mg, 0.02 mmol), K_3PO_4 (106 mg, 0.5 mmol), and diamine **5** (12 mg, 0.1 mmol). 1,4-dioxane (1 mL) was introduced through the septum. The resulting suspension was stirred under argon at 90 °C for 18 h and cooled. The mixture was adsorbed onto silica gel and purified by flash chromatography (CH_2Cl_2/MeOH/NH_4OH: 92/5/3) to afford the above product (70 mg, 88%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.46 (d, J = 5.0 Hz, 2H), 7.46 (d, J = 9.0 Hz, 2H), 7.31 (d, J = 2.5 Hz, 2H), 6.76 (dd, J = 2.5, 9.0 Hz, 2H), 6.32 (d, J = 5.0 Hz, 2H), 5.45 (s, 2H), 3.90 (s, 6H), 3.40 (q, J = 5.0 Hz, 4H), 2.87 (t, J = 5.5 Hz, 4H), 2.43 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 160.4, 151.5, 150.4, 149.8, 120.7, 117.0, 113.4, 108.5, 98.1, 77.5, 77.2, 77.0, 55.7, 55.5, 42.2, 40.3. FTIR (thin film, cm^{-1}): 2957, 1620, 1586, 1473, 1231, 1040. HRMS-ESI (m/z): calculated for $C_{25}H_{29}N_5O_2$ $[M + H]^+$: 432.2400, found: 431.2405.

Synthesis of Compound 11 (Lys04). A round-bottom flask was charged with 4-bromo-7-chloroquinoline **4** (734 mg, 3.0 mmol), $Pd(OAc)_2$ (23 mg, 0.1 mmol), BINAP (125 mg, 0.2 mmol), K_3PO_4 (1.06 g, 5.0 mmol), and 1,8-diamino-3,6-dioxaoctane **10** (148 mg, 1.0 mmol). Dioxane (10 mL) was introduced through the septum. The resulting suspension was stirred under argon at 90 °C for 18 h and cooled. The mixture was adsorbed onto silica gel and purified by flash chromatography (CH_2Cl_2/MeOH: 90/9/1) to afford the above product (400 mg, 85%) as a pale solid. Mp: 150–151 °C; R_f = 0.28 (silica gel, CH_2Cl_2/MeOH/NH_4OH: 90/9/1); ^{1}H NMR (500 MHz, CDCl3): δ 8.53 (d, J = 5.5 Hz, 2H), 7.92 (d, J = 2.0 Hz, 2H), 7.59 (d, J = 9.0 Hz, 2H), 7.24 (d, J = 2.0 Hz, 2H), 6.37 (d, J = 5.5 Hz, 2H), 5.39 (s, 2H), 3.85 (t, J = 5.0 Hz, 4H), 3.75 (s, 4H), 3.47 (q, J = 5.0 Hz, 4H). ^{13}C NMR (125 MHz, CDCl3): δ 152.0, 149.7, 149.1, 135.0, 128.9, 125.4, 121.0, 117.3, 99.3, 70.3, 68.8, 42.7. FTIR (thin film, cm^{-1}): 3,266, 3,066, 2,917, 1,611, 1,582. HRMS-ESI (m/z): calculated for $C_{24}H_{24}N_4O_2Cl_2$ $[M+H]^+$: 471.1355, found: 471.1357.

Synthesis of Compound 12 (Lys-05). To generate a water soluble salt of compound **3**, a suspension of compound **3** (896 mg, 2.04 mmol) in MeOH (40 mL) was treated with HCl gas for 10 min at 0 °C. The mixture was stirred for another 12 h at room temperature. The solvent was removed by rotary evaporation and the residue was dried under vacuum at 50 °C overnight to afford the salt **12** (1.13 g, 100%) as a yellow solid. Mp: 270 °C (decomposition); ^{1}H NMR (500 MHz, D_2O): δ 8.12 (d, J = 7.0 Hz, 2H), 7.73 (d, J = 9.0 Hz, 2H), 7.58 (d, J = 2.0 Hz, 2H), 7.26 (dd, J = 9.0, 2.0 Hz, 2H), 6.62 (d, J = 2.0 Hz, 2H), 3.89 [broad multiplet (br), 4H], 3.68 (br, 4H), 3.12 (s, 3H). ^{13}C NMR (125 MHz, D_2O): δ 155.8, 142.8, 140.2, 137.2, 128.1, 123.8, 119.1, 114.8, 98.7, 52.9, 42.7, 38.2. FTIR (thin film, cm^{-1}): 3,376, 3,019, 2,914, 1,631, 1,612, 1,215. HRMS-ESI (m/z): calculated for $C_{23}H_{24}N_5Cl_2$ $[M - 3HCl + H]^+$: 440.1409, found: 440.1408.

Scoring for Paneth Cell Dysfunction and Lysozyme Staining. Scoring for histological analysis was conducted as follows: intestinal samples were included in the analysis if they consisted of terminal ileum, and excluded if they consisted predominantly of colon. Using the 40× and 20× objectives, for each sample the number of Paneth cells per intestinal crypt were counted for 10 continuous crypts. Paneth cell dysfunction was scored by assigning a score for each Paneth cell in 10 continuous crypts that characterized the number and size of eosinophilic secretory granules: 0, normal number and size; 1, normal number, reduced size; 2, reduced number, normal size; and 3, reduced number, reduced size. A segment from the distal small intestine 1 cm from the cecum was isolated from the treated and control mice, rinsed in ice-cold PBS, fixed, and embedded; immunohistochemistry against lysozyme was then performed as described (1, 2). Lysozyme-positive Paneth cells were counted using lysozyme immunofluorescence to visualize the Paneth cell granules, and β-catenin costain to identify cell edges.

Measurement of Lyso5 and HCQ Using HPLC Tandem Mass Spectrometry. Standard curves were constructed from stock concentrations of 100 μM HCQ and Lys05 in the appropriate buffers. Samples were diluted with corresponding buffer to a protein concentration of 2 μg/μL and prepared in duplicate by protein precipitation. Briefly, 400 μL was dispensed into wells in a 96-well plate (Isolute PPT+; Biotage), followed by the addition of 100 μL of sample. The plate was allowed to stand for 5 min and then subjected to −15 inches Hg of vacuum pressure for 6 min. Extracts were dried under a stream of nitrogen gas and reconstituted in 100 μL of 0.1% formic acid/15% methanol/water. A total of 10 μL was injected onto the LC column for mass spectral analysis using a cooled autosampler. Chromatographic separation was performed using a 2.0 mm (internal diameter) × 100 mm Synergi 2.5 U Polar-RP column (Phenomenex) at a 0.2-mL flow rate and gradient elution with 0.1% formic acid/methanol and 0.1% formic acid/water. Quantitation was achieved using a Varian 1200L system operated in positive ESI mode using multiple-reaction monitoring. Lys05 and HCQ were monitored using specific precursor ion to product ion transitions of m/z 440.2 → 205 and 336.2 → 247, respectively.

1. Crissey MA, Guo RJ, Fogt F, et al. (2008) The homeodomain transcription factor Cdx1 does not behave as an oncogene in normal mouse intestine. *Neoplasia* 10:8–19.

2. Crissey MA, et al. (2011) Cdx2 levels modulate intestinal epithelium maturity and Paneth cell development. *Gastroenterology* 140:517–528.

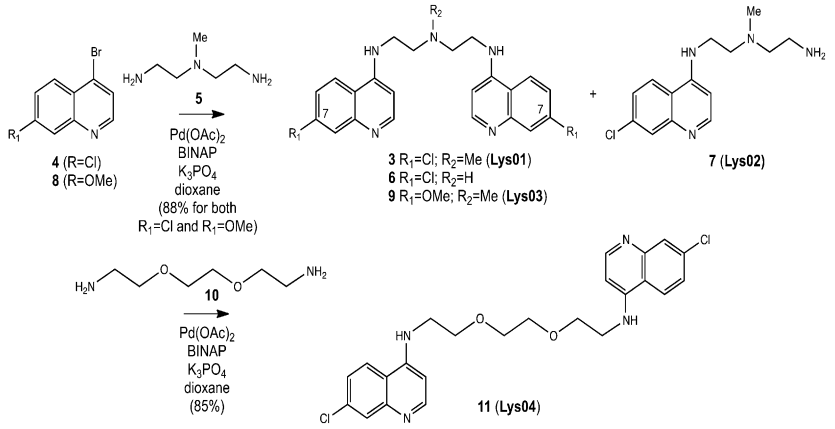

Fig. S1. Synthesis schema for bisaminoquinolines.

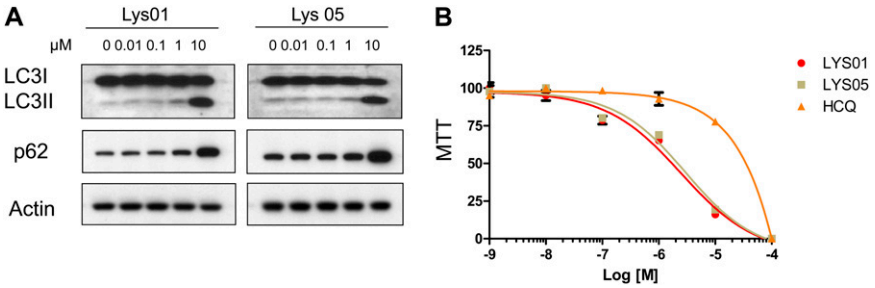

Fig. S2. Autophagy inhibition and cytotoxicity of Lys05, the water-soluble salt of Ly01. (*A*) Immunoblotting against LC3 and p62 in c8161 cells treated as indicated. (*B*) A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in c8161 cells at 72 h. HCQ, hydroxychloroquine. Values presented are mean ± SD with five replicates per treatment condition.

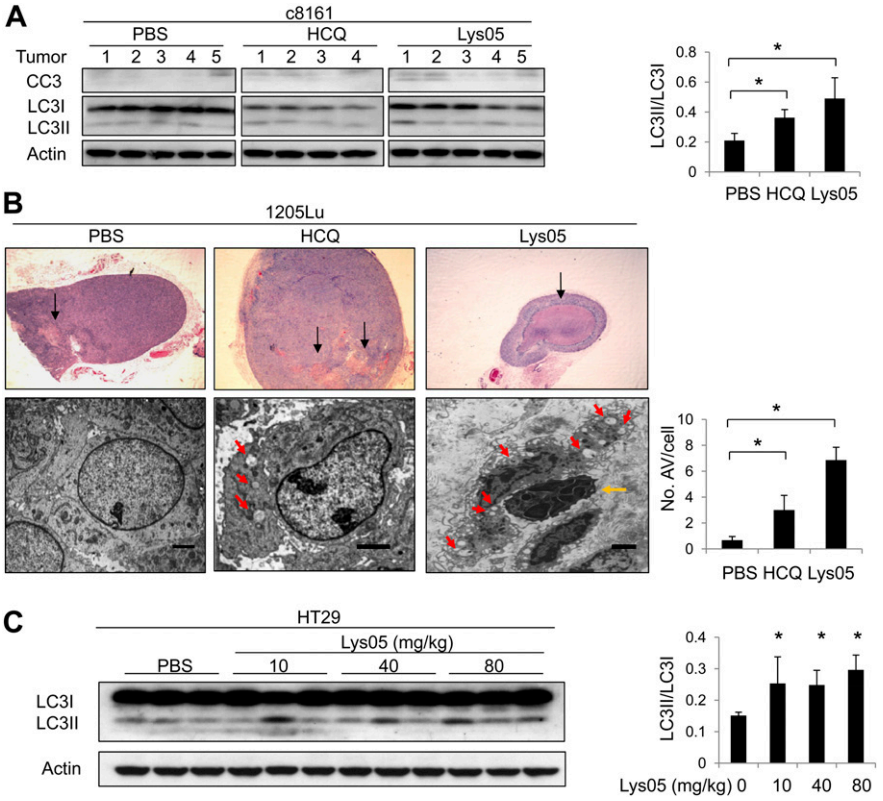

Fig. S3. Autophagy inhibition and tumor necrosis in melanoma and colon cancer xenografts treated with Lys05 or HCQ. (*A*) Immunoblotting against LC3 in lysates from individual c8161 tumors treated as indicated with daily i.p. injections for 48 h. Quantification of LC3II/Lc3Iratio (mean ± SEM). (*B*) Tumor necrosis (arrows) in H&E-stained sections of 1205Lu tumor xenografts harvested after 14 d of treatment. Electron micrographs (7,000–12,000×) of melanoma tumor cells. Arrows: autophagic vesicle (white); apoptotic cell (orange). (*C*) Immunoblotting against LC3 in HT29 xenografts treated with daily dosing (10 and 40 mg/kg) or 3/5 d (80 mg/kg) for 14 d.

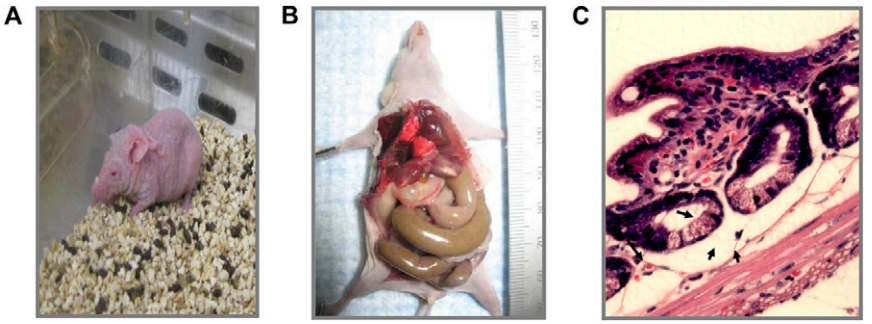

Fig. S4. Toxicity associated with Lys05 76 mg/kg i.p. 3/5 d. (*A*) Mice were lethargic with arched backs after 3 d of dosing. (*B*) 3/10 mice developed bowel obstruction. (*C*) Dysmorphic Paneth cells (arrows) in the terminal ileum of one mouse.

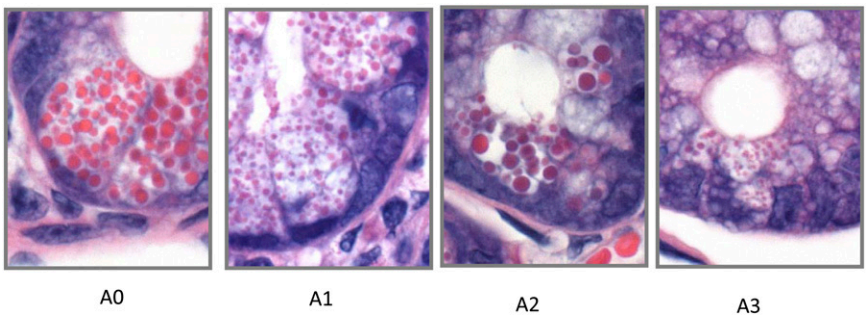

Fig. S5. Paneth cell dysfunction scale. Under 40× power the size and number of eosinophilic granules per Paneth cell was scored for 10 Paneth cells per sample: A0, normal size and number; A1, decreased size, normal number; A2, normal size, decreased number; and A3, decreased size and number.

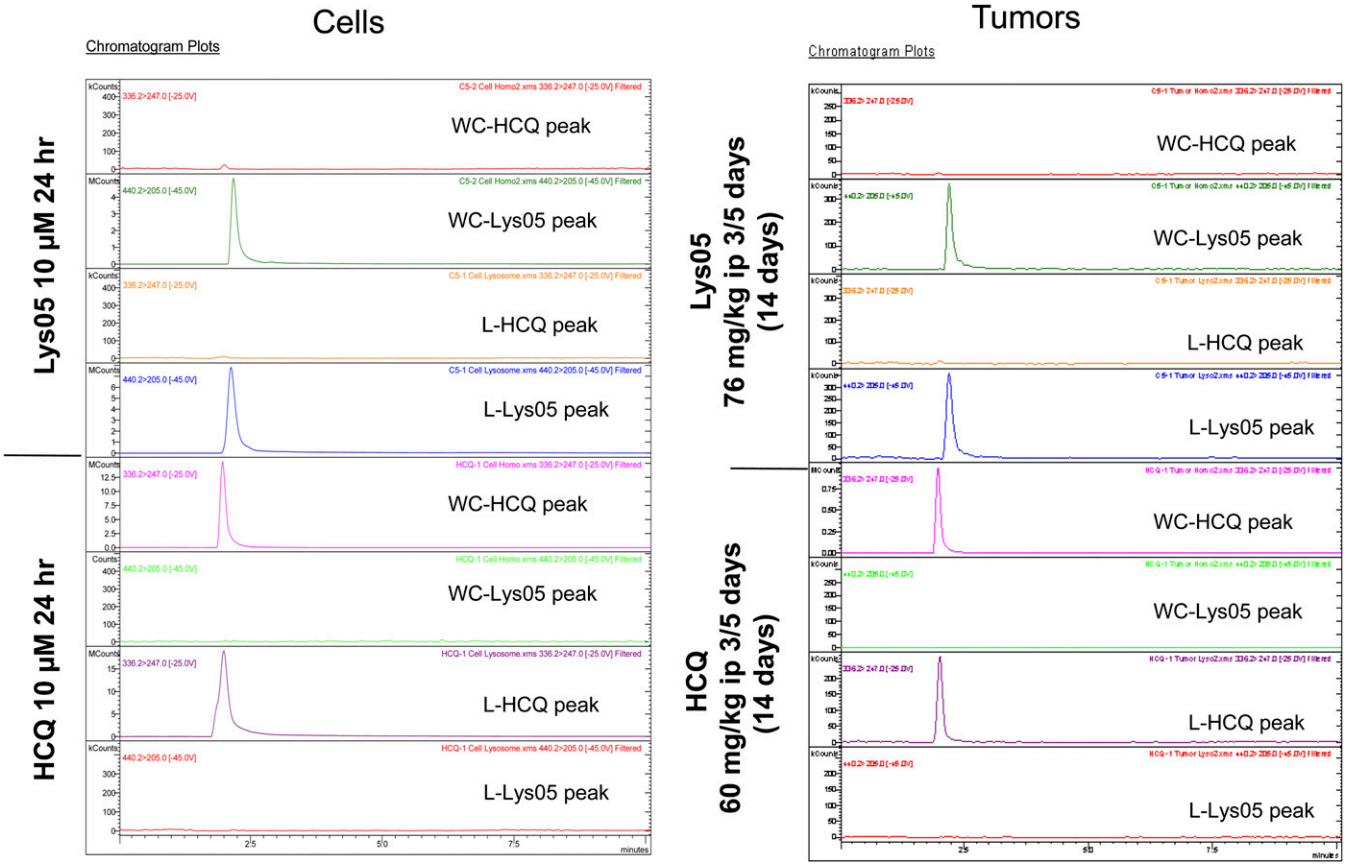

Fig. S6. HPLC tandem mass spectrometry assay for HCQ and Lys05. 1205Lu cells (24 h) and 1205Lu tumors (14 d). WC, whole-cell homogenate; L, lysosomal subfraction; HCQ, hydroxychloroquine.

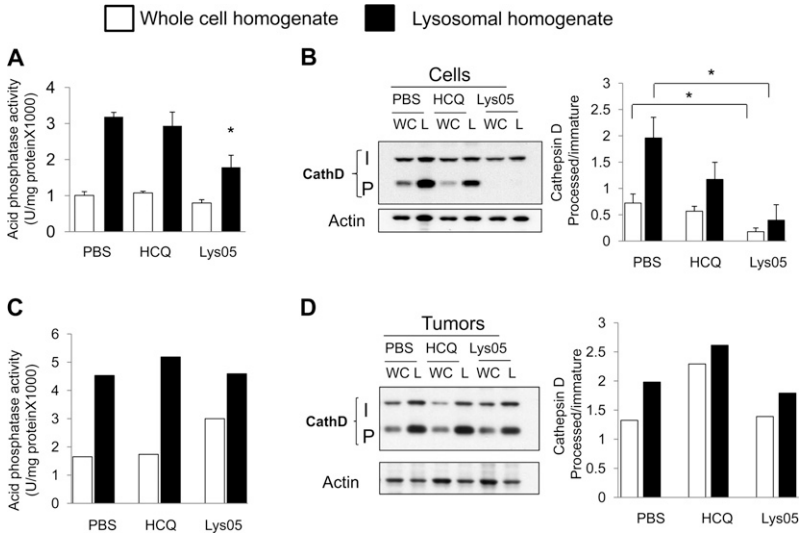

Fig. S7. Impairment of lysosomal enzymes and extralysosomal leakage associated with Lys05 treatment. (*A*) Acid phosphatase activity and (*B*) cathepsin D immunoblotting in whole cell (white, WC) and lysosomal (black, L) fractions of 1205Lu cells treated with PBS, HCQ 10 μM, or Lys05 10 μM for 24 h. Graphs show the mean ± SEM for three independent experiments. (*C*) Acid phosphatase activity and (*D*) cathepsin D immunoblotting in whole-cell (WC, white) and lysosomal (L, black) fractions of 1205Lu xenografts treated with PBS, HCQ 60 mg/kg, or Lys05 76 mg/kg i.p. 3/5 d (tumors). WC and L homogenates prepared from three separate tumors were pooled together. $*P < 0.05$.

Table S1. Compounds

Compound no.	Name	IUPAC nomenclature
1	Chloroquine	(*S*)-N^4-(7-chloroquinolin-4-yl)-N^1,N^1-diethylpentane-1,4-diamine
2	Hydroxychloroquine	*(RS)*-2-[{4-[(7-chloroquinolin-4-yl)amino]pentyl}(ethyl)amino]ethanol (*S*)-2-((4-((7-chloroquinolin-4-yl)amino)pentyl)(ethyl)amino)ethanol
3	Lys01	N^1-(7-chloroquinolin-4-yl)-N^2-(2-((7-chloroquinolin-4-yl)amino)ethyl)-N^2-methylethane-1,2-diamine
4	—	4-bromo-7-chloroquinoline
5	—	N^1-(2-aminoethyl)-N^1-methylethane-1,2-diamine
6	—	N^1-(7-chloroquinolin-4-yl)-N^2-(2-((7-chloroquinolin-4-yl)amino)ethyl)ethane-1,2-diamine
7	Lys02	N^1-(2-aminoethyl)-N^2-(7-chloroquinolin-4-yl)-N^1-methylethane-1,2-diamine
8		4-bromo-7-methoxyquinoline
9	Lys03	N^1-(7-methoxyquinolin-4-yl)-N^2-(2-((7-methoxyquinolin-4-yl)amino)ethyl)-N^2-methylethane-1,2-diamine
10		2,2′-(ethane-1,2-diylbis(oxy))diethanamine
11	Lys04	*N*,*N*′-((ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))bis(7-chloroquinolin-4-amine)
12	Lys05	N^1-(7-chloroquinolin-4-yl)-N^2-(2-((7-chloroquinolin-4-yl)amino)ethyl)-N^2-methylethane-1,2-diamine trihydrochloride

IUPAC, International Union of Pure and Applied Chemistry.

Table S2. IC_{50} (μM) of aminoquinolines

Cell line	Malignancy	Lys01	Lys02	Lys03	Lys04	HCQ
1205Lu	Melanoma	3.6	90.7	41.4	10.4	23.8
c8161	Melanoma	3.8	80.0	23.6	17.0	42.0
LN229	Glioma	7.9	45.0	52.5	14.9	29.9
HT-29	Colon	6.0	35.0	19.4	11.1	15.6