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Synthesis of quinoline thioethers as novel small molecule enhancers of monoclonal antibody production in mammalian cell culture

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ABSTRACT

A small library of novel quinoline derivatives containing different thioether substituents at position 4 have been synthesized and screened in murine hybridoma cell culture for their ability to enhance monoclonal antibody (mAb) production. From this set of compounds, four compounds have been discovered that enhanced immunoglobulin G (IgG) titer over negative control cultures due to an increase in specific productivity. These results demonstrate the utility of using organic synthesis and parallel ligand screening methods to discover novel cell culture additives that may be useful for increasing mAb yield in industrial biomanufacturing processes.

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1. Introduction

The ability of inexpensive low molecular weight compounds (LMWCs) to enhance monoclonal antibody (mAb) yield in mammalian cell culture would be immensely beneficial in industrial biomanufacturing operations and other end-user applications. In particular, the discovery and deployment of suitable LMWCs could lead to improved large-scale production efficiencies, enhancements to product development, and better cost effectiveness. Several different low molecular weight compounds that enhance mAb titer in mammalian cell culture are already known, including histone deacetylase inhibitors, such as sodium butyrate,[1] valproic acid, and other aliphatic carboxylate analogues,[2] the mTOR inhibitor, rapamycin,[3] the chemical chaperones 4-phenyl-butyric acid and other aromatic carboxylic acids,[4] DNA methyltransferase inhibitors, such as hexano-hydroxamic acid; as well as dimethylsulfoxide and retinoic acid.[5]

Combinatorial or parallel synthesis using solid- and liquid-phase approaches to prepare low molecular weight organic molecules based on known pharmacophores has evolved in recent years to become a powerful tool for the development of chemical libraries of novel lead compounds in drug discovery.[6] However, the screening of such synthetic libraries for the discovery of novel cell culture additives for the production of recombinant proteins has, to the best of our knowledge, not been reported. Such ligand discovery and screening methods could provide an avenue to identify new classes of compounds having the desired and more potent functionalities, and which may act synergistically with known chemical additives.

Among the broad range of pharmacophores available for drug development are nitrogen heterocycle scaffolds, containing different functional groups that can be selectively modified by organic synthesis to generate libraries consisting of a diverse range of derivatives.[7] One such nitrogen heterocycle scaffold is quinoline, a rigid planar molecule, that is, a pharmacophore present in the core of numerous physiologically active agents that display interesting therapeutic properties by acting as either enzyme inhibitors or as ligands for cell surface receptors.[8]

Structurally, quinoline can be readily modified with a broad range of substituent groups to provide the molecular diversity necessary to achieve a library of compounds whereby different members can show different biological effects.[9] As part of our effort to discover novel low molecular weight compounds, which enhance the yield of expressed recombinant proteins, including genetically engineered monoclonal antibodies (mAbs), using mammalian cell culture, we have used quinoline as a scaffold to synthesize a small library of derivatives containing different thioether substituents at the 4-position, and a *N*-(2-(2-(2-amino-ethoxy)ethoxy)ethyl)carboxamide group at the 2-position to improve water solubility (Fig. 1). Each member of this chemical library was then screened to evaluate their effect on immunoglobulin G (IgG) mAb titer and cell viability using two murine hybridoma cell lines of different clonal

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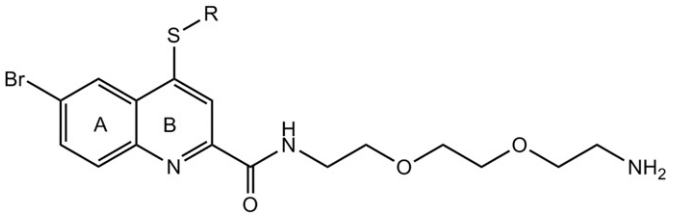


Fig. 1. General structure of the low molecular weight quinoline thioether compounds, whereby R could be alkyl, aryl, alkylamino, carboxylmethyl, etc.

ancestry. Four of the compounds investigated significantly enhanced the IgG titer, mainly by enhancing specific productivity. These findings clearly demonstrate the potential of using molecules derived from such approaches to enhance the yield of mAbs and other recombinant proteins in research and industrial biomanufacturing processes.

2. Results and discussion

2.1. Chemistry

As part of our earlier SAR design approaches, 6-bromo-4-chloroquinolinic acid methyl ester (**5**) was identified as a suitable, synthetically accessible, diversity scaffold that could be used to generate a library of structures for bioactivity screening (Scheme 1).

To synthesize this scaffold, 4-bromoaniline **1** was first reacted with dimethyl acetylenedicarboxylate (DMAD) **2** to give the Michael adduct, dimethyl anilinofumarate, **3** in excellent yield on multigram scales. We initially attempted to convert the Michael adduct **3** to the 4-(1*H*)-quinolone **4** by a thermal Conrad–Limpach method[10] involving refluxing **3** in diphenyl ether. However, under a variety of conditions involving both conventional heating and microwave techniques with different solvents, contact times and reaction temperatures, these methods invariably gave viscous tars containing an unsatisfactory yield of the desired product that was difficult to purify. To overcome these problems, advantage was taken of solventless microwave techniques since the starting material was a liquid. Under these conditions, ring closure to the enamine **3** readily occurred.

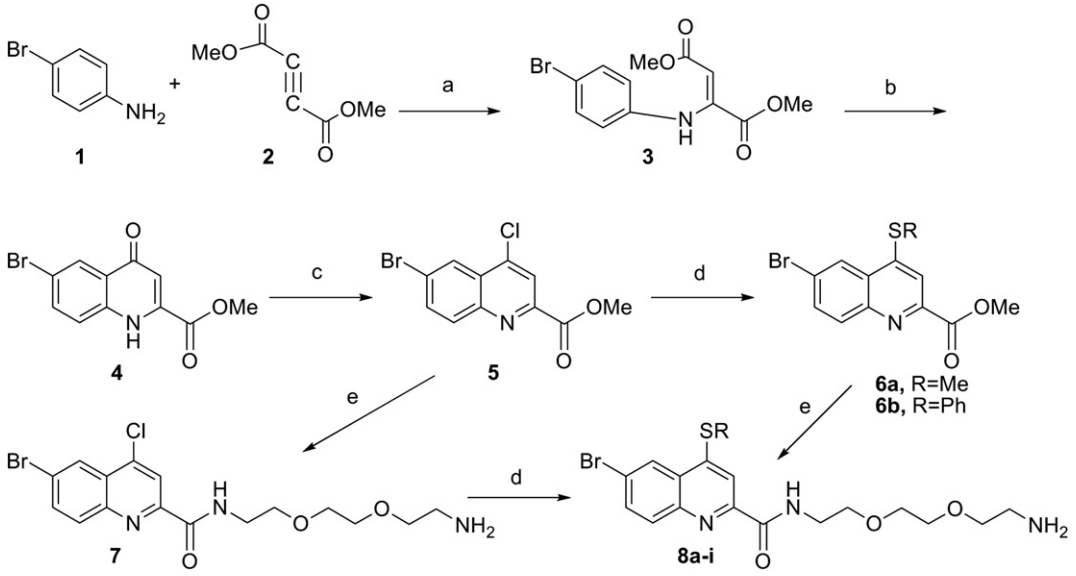
The yield from this reaction was moderate (40%), leaving behind mainly starting material, presumably as a consequence of non-uniform heat distribution affecting product formation during the microwave process. Finally, polyphosphoric acid[11] (PPA) was chosen as both a catalytic cyclization reagent and solvent, and gave the 4(1*H*)-quinolone **4** at lower temperature and in greater yield (78%). The desired product was easily purified on multigram scales by simply washing with water. The intermediate **4** was then chlorinated with phosphorous oxychloride to give the desired quinoline analogue as the diversity scaffold **5** in excellent yield.

Since the quinoline core is poorly water soluble, we introduced a hydrophilic *N*-(2-(2-(2-amino-ethoxy)ethoxy)ethyl)-carboxamide group at position 2 to improve water solubility and biocompatibility. This modification was achieved by reacting the ester **5** with a small excess of 2, 2′-(ethylene-dioxy)-bis-ethyl-amine. Although mono-acylation of diamines is usually difficult to accomplish, we obtained an excellent transformation (98%) at room temperature by employing *p*-toluenesulfonic acid as a catalyst. This procedure eliminated the need for a less efficient deprotection and activation of the masked carboxylic acid, and is both cost-effective and scalable.

For our initial screening studies for novel cell culture additives we chose to introduce diversity at position 4 by nucleophilic substitution of the chloride with different commercially available thiols to generate a small library **8a**–**i** (Fig. 2). Alternatively, **5** could also be thiolated and the ester subsequently substituted with 2,2′-(ethylenedioxy)-bis-ethylamine to give the final product. However, this route was found to be more chromatographically intensive. Therefore most of the library members were prepared from **7** (Scheme 1). It can be noted that the quantitative ^{19}F NMR spectroscopy of the various purified synthetic molecules revealed that several compounds, e.g., **8b**, **8c**, **8d**, **8f**, and **8h**, occurred as the corresponding stoichiometric TFA salts despite extensive lyophilisation procedures being employed following their reversed phase chromatographic purification. The occurrence of these compounds as their TFA salts is consistent with the chemical shift differences of the terminal methylene at δ=3.08 ppm for the TFA salt compared to the free base at δ=2.80 ppm.

2.2. Cell culture procedures

The effect of the quinoline compounds on IgG titer and cell viability was measured at four different concentrations from 80 nM to 100 μM. Of the compounds tested, **8b**, **8c**, and **8d** (as their trifluoracetate salts) at 80 nM increased the IgG titer from clone A by



Scheme 1. Reagents and conditions: (a) MeOH, reflux, 2 h, 97%; (b) PPA, 130 °C, 1 h, 78%; (c) $POCl_3$, 100 °C, 1.5 h, 95%; (d) NaSR, DMF, rt, 2–12 h; (e) 2,2′ethylene dioxy-bis-ethylamine, PTSA, MeOH, 24 h, 84%.

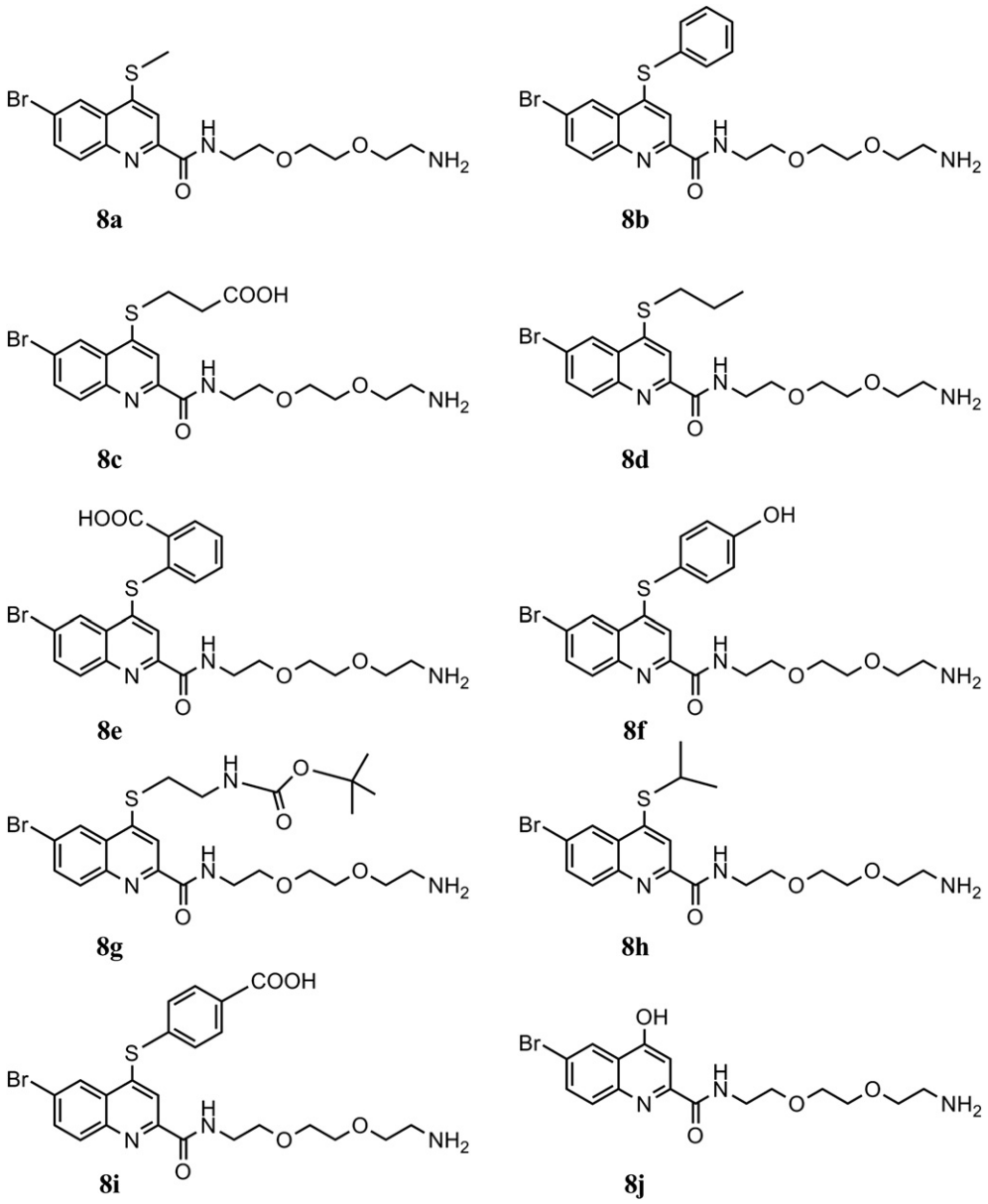


Fig. 2. List of quinoline compounds discussed in the test.

1.2–1.4-fold, relative to the control culture (Fig. 3A), but did not enhance IgG production from clone B, whilst compound **8h** increased IgG titer from clone B by 1.2-fold at 80 nM concentration (Fig. 3B). The remaining compounds, tested at the same concentration, did not enhance IgG production from either clone. In general, the test compounds either had no significant effect ($p>0.05$) on cell viability or slightly reduced cell viability at both concentrations (Fig. 3).

These findings demonstrate that the significant increases in IgG titer elicited by **8b**, **8c**, **8d**, and **8h** can be attributed to enhanced specific productivity (in terms of nmol IgG/cell/min) by both clones. Further increasing the concentration of the other test compounds as their trifluoracetate salts to 1 μM did not increase IgG production by either clone. Compound **8d** elicited a 2.2-fold enhancement of IgG titer from clone A at 0.8 μM (Fig. 3A) whilst **8h** increased IgG titer 1.4-fold from clone B at 0.8 μM (Fig. 3B). Both of these increases were also due to enhanced specific productivity. Higher concentrations of the library compounds (10 μM and 100 μM) were cytotoxic in these bioassays (data not shown).

Low molecular weight cell culture additives that increase the yield of recombinant proteins, including mAbs, from cultured mammalian cells would have significant utility in the biomanufacturing industry. As noted above, previous studies on the effect of low molecular weight compounds on mAb production have largely focused on compounds with known bioactivity, e.g., histone deacetylase, DNA methyltransferase and mTOR inhibitors, or their analogues, where productivity enhancements in similar ranges to those determined in the current investigations have been observed. An alternative approach to discover novel compounds that enhance mAb production is to screen synthetic compound libraries built around known pharmacophores. Here, we have synthesized and screened members of a small library of quinoline derivatives, a pharmacophore with a range of known bioactivities, to discover several novel compounds that enhance mAb production at the 80 nM level.

The quinoline derivatives synthesized in this study fell into three broad categories, namely aliphatic, aromatic, and carboxylic thioether substituents at the 4 position. In general, the compounds that

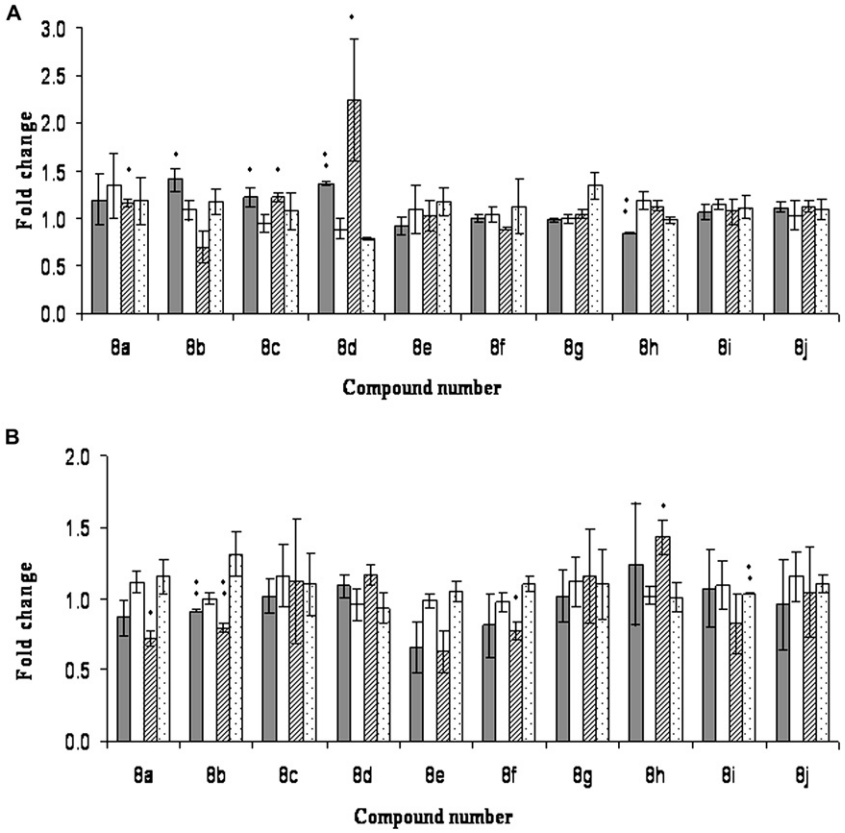


Fig. 3. Effect of quinoline compounds on IgG titer (80 nM, gray and 0.8 μM, diagonal columns) and cell viability (80 nM, open and 0.8 μM, dotted columns). Data are presented as the mean percent of control from three replicates±S.D. $^{*}p<0.05$; $^{**}p<0.01$; (A). From clone A culture; (B) From clone B culture.

enhanced IgG titer were those bearing a hydrophobic aliphatic or unsubstituted aromatic thiother substituent, with the propyl-containing derivative **8d** giving a 2.2-fold enhancement with clone A. The exception to this trend was compound **8c**, which contained a carboxylic acid group at the end of the propyl chain. It is also clear from this work that hybridoma cell lines of different clonal ancestry are affected differently by cell culture additives. Therefore in an industrial setting it would be prudent to screen potential novel cell culture additives against different cell lines to determine the best producers and conditions for optimal and maximal mAb production.

3. Conclusion

In conclusion, we have designed a simple synthetic route toward a new series of 4-thio-substituted quinoline derivatives with different side chain substituents These compounds have been subjected to cell-based assays to investigate their effects on the production of immunoglobulin G (IgG) mAb by two murine hybridoma cell lines of different clonal ancestry. From this study we have identified several novel additives that enhance mAb production by up to 2.2-fold with specific hybridoma cell lines. These results clearly demonstrate the utility of using organic synthesis to generate compound libraries to discover novel active, cell culture additives to improve productivity and yields of expressed recombinant proteins in biomanufacturing processes and value-add to the final production technology. Implementation of such technologies would deliver economic benefits through increased productivity in large-scale cell culture procedures, in traditional bioreactor or wave-bag format, with concomitant reduction of manufacturing costs in unit batch operations. Based on the above results, future studies related to the introduction of structural diversity around the A ring of **8** are anticipated to lead to even greater enhancements in expression productivity at levels below 100 nM with reduced cytotoxity at higher concentrations.

4. Experimental section

4.1. General

All reagents and solvents were purchased from Sigma–Aldrich, Acros Organics or Alfa Aesar and used without further purification. TLC was performed on Merck silica gel 60 F_{254} precoated aluminum plates. The components were visualized by fluorescence under 254 nm ultraviolet irradiation or by exposure to a variety of development/charring reagents where necessary.

Microwave-assisted reactions were carried out in a CEM-Explorer Automated Microwave Workstation with temperatures measured by an online IR sensor. Flash chromatography was performed with a FlashMaster II instrument using Merck silica gel 60, 0.040–0.063 μm (230–400 mesh) for normal phase and Grace Vydac C18 silica gel (Davisil), 35–70 μm for reverse phase chromatography using the automated gradient elution mode.

The ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a Bruker DPX-300 spectrometer (300 MHz ^{1}H, 75 MHz ^{13}C) and Bruker DPX-400 spectrometer (400 MHz ^{1}H, 100.6 MHz ^{13}C). The ^{1}H NMR spectra refer to solutions in deuterated solvents as indicated. The solvent peaks were used as an internal reference, except $CDCl_3$ where tetramethylsilane (TMS) was used as the internal standard (δ 0.00 ppm). The ^{19}F NMR spectra were acquired as solutions in deuterated solvents as indicated and $CFCl_3$ (trichloro-fluoromethane) used as the internal standard (δ 0.00 ppm). Since automated NMR techniques were used, the peak for residual H_2O/CD_3OH was automatically referenced to 4.87 ppm. Because the chemical shift of this signal is sensitive to the presence of acidic and

basic compounds the signal due to CHD_2OD did not always appear at ~3.30 ppm. Accordingly, the ^1H NMR data for all samples recorded in CD_3OD were corrected using the CHD_2OD signal as internal reference. Infrared spectra were recorded on a Perkin–Elmer Spectrum RXI Fourier Transform infrared spectrometer as KBr disks of solids or thin films of liquid (neat) between sodium chloride plates. Low resolution electrospray ionisation mass spectra (ESI) were recorded on a Micro mass Platform II API QMS Electrospray mass spectrometer with cone voltage at 25 V as solutions in MeOH unless otherwise indicated. HRMS were recorded with an Agilent 6220 Accurate Mass TOF LC/MS spectrometer in the positive (ESI^+) mode. During the course of these studies it was noted that calculations based on the ChemDraw and the website http://www-jmg.ch.cam.ac.uk/tools/magnus/MolWeight.html software give different values for exact masses from the third decimal place. Calculated exact masses of the various compounds reported below were derived with the ChemDraw software.

Melting points were determined using a Stuart melting point apparatus (SMP3) with digital thermometer and are uncorrected.

*4.1.1. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-chloroquinoline-2-carboxamide (**7**).* To a stirred solution of methyl 6-bromo-4-chloroquinoline-2-carboxylate **5** (208 mg, 0.692 mmol) in dry methanol/chloroform, 2:1 (5 mL) were added 2,2′-ethylene dioxy-bis-ethylamine (321 mg, 2.166 mmol) and a catalytic amount of *p*-toluenesulfonic acid (10 mg). The resulting solution was stirred at room temperature for 24 h and then concentrated in vacuo. The residue was diluted with saturated $NaHCO_3$ (50 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by reverse phase flash chromatography (acetonitrile/water, 1:1) to give *N*-(2-(2-(2-aminoethoxy) ethoxy) ethyl)-6-bromo-4-chloroquinoline-2-carboxamide (245 mg, 84%) as a yellow solid. MP: 97.5–98.8 °C; IR (KBr) ν_{max}: 3370, 2869, 1663, 1527, 1482, 1305, 1136, 983, 927, 889, 830 cm^{-1}; ^1H NMR (MeOD): δ 2.75 (t, *J*=5.3 Hz, 2H), 3.52 (t, *J*=5.3 Hz, 2H), 3.63–3.75 (m, 8H), 7.98 (dd, *J*=8.9, 2.1 Hz, 1H), 8.06 (d, *J*=8.9 Hz, 1H), 8.25 (s, 1H), 8.41 (d, *J*=2.1 Hz, 1H). ^{13}C NMR (MeOD): δ 40.8, 42.3, 70.7, 71.5, 71.6, 73.6, 120.9, 125.0, 127.5, 129.5, 133.2, 136.1, 144.1, 147.5, 151.7, 165.4; HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{16}H_{20}BrClN_3O_3$: 416.0371, found 416.0369.

*4.1.2. Synthesis of methyl 6-bromo-4-(methylthio)-quinoline-2-carboxylate (**6a**).* To a stirred solution of methyl 6-bromo-4-chloroquinoline-2-carboxylate **5** (1.0 g, 3.328 mmol) in dry DMF (10 mL) at room temperature was slowly added sodium thiomethoxide (231 mg 3.296 mmol). The resulting suspension was stirred for 3 h at room temperature and the reaction was then quenched with water (50 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with water (2×50 mL), brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash chromatography (EtOAc/hexane, 1:1) to give methyl 6-bromo-4-(methylthio)-quinoline-2-carboxylate (876 mg, 85%) as an off-white solid. MP: 172.8–173.5 °C; IR (KBr) ν_{max}: 1714, 1560, 1452, 1331, 1243, 1049 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.70 (s, 3H), 4.08 (s, 3H), 7.84 (d, *J*=8.7 Hz, 1H), 7.94 (s, 1H), 8.11 (d, *J*=8.9 Hz, 1H), 8.29 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 14.2, 53.4, 115.3, 122.9, 125.8, 128.3, 132.8, 134.1, 145.1, 147.3, 149.9, 165.9; HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{12}H_{11}BrNO_2S$: 311.9688, found 311.9691.

*4.1.3. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(methylthio)quinoline-2-carboxamide (**8a**).* To a stirred solution of methyl 6-bromo-4-(methylthio)-quinoline-2-carboxylate **6a** (80 mg, 0.256 mmol) in dry methanol (5 mL) were added 2,2′-ethylene dioxy-bis-ethylamine (114 mg, 0.769 mmol) and catalytic amount of *p*-toluenesulfonic acid (10 mg). The resulting solution was stirred at 60 °C for 24 h before concentrated in vacuo. The residue was diluted with saturated $NaHCO_3$ (50 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography (MeOH/EtOAc, 1:1 with 1% Et_3N) to give *N*-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(methyl-thio)quinoline-2-carboxamide (83 mg, 76%) as a pale yellow semi-solid; IR (KBr) ν_{max}: 3388, 2868, 1676, 1526, 1484, 1427, 1392, 1306, 1119, 826 cm^{-1}; ^1H NMR (MeOD): δ 2.64 (s, 3H), 2.77 (t, *J*=5.3 Hz, 2H), 3.53 (t, *J*=5.3 Hz, 2H), 3.63–3.75 (m, 8H), 7.72 (dd, *J*=9.0, 1.9 Hz, 1H), 7.79 (d, *J*=9.0 Hz, 1H), 7.84 (s, 1H), 8.03 (d, *J*=1.7 Hz, 1H); ^{13}C NMR (MeOD): δ 14.4, 40.8, 42.3, 70.8, 71.5, 71.6, 73.5, 114.1, 123.0, 126.7, 129.1, 133.1, 134.9, 145.5, 150.4, 151.5, 166.5; HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{17}H_{23}BrN_3O_3S$: 428.0643, found 428.0640.

*4.1.4. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(phenylthio)quinoline-2-carboxamide trifluoroacetate salt (**8b**).* To a stirred solution of methyl 6-bromo-4-(phenylthio) quinoline-2-carboxylate **6b** (187 mg, 0.50 mmol) in dry MeOH (5 mL) were added 2,2′-ethylene dioxy-bis-ethylamine (222 mg, 1.498 mmol) and a catalytic amount of *p*-toluenesulfonic acid (20 mg). The resulting solution was stirred at 60 °C for 24 h and then concentrated in vacuo. The residue was diluted with saturated $NaHCO_3$ (50 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by reverse phase flash chromatography (acetonitrile/water, 4:1 with 1% TFA) as eluent to give *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-bromo-4-(phenylthio)-quinoline-2-carboxamide (176 mg, 59%, as the TFA salt)[12] as an off-white semi-solid; IR (KBr) ν_{max}: 3387, 2927, 1678, 1528, 1483, 1431, 1205, 1131, 834, 800, 722 cm^{-1}; ^1H NMR (MeOD): δ 3.07 (t, *J*=5.0 Hz, 2H), 3.59 (t, *J*=5.1 Hz, 2H), 3.63–3.73 (m, 8H), 7.54–7.61 (m, 3H), 7.60 (s, 1H), 7.62–7.66 (m, 2H), 7.94 (dd, *J*=8.9, 1.7 Hz, 1H), 8.03 (d, *J*=8.9 Hz, 1H), 8.42 (d, *J*=1.8 Hz, 1H); ^{13}C NMR (MeOD): δ 40.5, 40.8, 68.0, 70.7, 71.5(×2), 116.8, 123.5, 126.9, 128.8, 129.7, 131.6, 131.7(×2), 133.3, 135.4, 136.7(×2), 146.0, 150.5, 151.3, 166.2; ^{19}F NMR (DMSO-d_6): δ −73.12 (s); HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{25}BrN_3O_3S$: 490.0800, found 490.0801.

*4.1.5. Synthesis of 3-(2-(2-(2-(2-aminoethoxy)ethoxy)ethyl-carbamoyl)-6-bromoquinolin-4-ylthio)propanoic acid trifluoroacetate salt (**8c**).* To a stirred solution of 3-mercaptopropionic acid (106 mg, 1.0 mmol) in dry THF (15 mL) at 0 °C was slowly added sodium hydride (72 mg, 3.0 mmol). The resulting suspension was stirred for 1 h at room temperature and then evaporated to dryness. The resulting white solid was slowly added to a stirred solution of *N*-(2-(2-(2-aminoethoxy)ethoxy) ethyl)-6-bromo-4-chloroquinoline-2-carboxamide **7** (208 mg, 0.499 mmol) in dry DMF (10 mL) and the mixture was stirred for 2 h at room temperature. The reaction was quenched with distilled water (50 mL), concentrated in vacuo, and the residue was purified by reverse phase flash chromatography (acetonitrile/water, 1:1 with 1% TFA) to give 3-(2-(2-(2-(2-aminoethoxy)ethoxy)ethylcarbamoyl)-6-bromoquino- lin-4-ylthio)propionic acid (175 mg, 58%, as the TFA salt)[12] as a pale yellow semi-solid; IR (KBr) ν_{max}: 3440, 2920, 1671, 1590, 1484, 1426, 1310, 1144, 1006, 826, 638 cm^{-1}; ^1H NMR(MeOD): δ 2.65 (t, *J*=7.3 Hz, 2H), 3.07 (t, *J*=5.1 Hz, 2H), 3.45 (t, *J*=7.3 Hz, 2H), 3.66–3.76 (m, 10H), 7.80 (dd, *J*=9.0, 2.1 Hz, 1H), 7.90 (d, *J*=9.0 Hz, 1H), 8.01 (s, 1H), 8.15 (d, *J*=2.0 Hz, 1H); ^{13}C NMR (MeOD): δ 29.1, 37.5, 40.6, 40.8, 68.3, 71.1, 71.5, 71.6, 115.0, 123.0, 127.0, 129.5, 133.3, 135.1, 145.8, 150.4, 150.7, 166.7, 179.0; ^{19}F NMR (DMSO-d_6): δ −73.56 (s); HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{19}H_{25}BrN_3O_5S$: 486.0698, found 486.0697.

*4.1.6. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(propylthio)quinoline-2-carboxamide trifluoroacetate salt (**8d**).* To

a stirred solution of propane thiol (76 mg, 1.0 mmol) in dry methanol (5 mL) was slowly added sodium methoxide (1 mL, 25% w/w). The resulting suspension was stirred for 1 h at room temperature and then *N*-(2-(2-(2-aminoethoxy)ethoxy) ethyl)-6-bromo-4-chloroquinoline-2-carboxamide **7** (209 mg, 0.502 mmol) in dry methanol (2 mL) was slowly added to the suspension. The mixture was stirred for 4 h at room temperature and the reaction was then quenched with water (50 mL), concentrated in vacuo and the residue extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by reverse phase flash chromatography (acetonitrile/water, 1:1 with 1% TFA) to give *N*-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(propylthio) quinoline-2-carboxamide (142 mg, 50%, as the TFA salt)[12] as a brown semi-solid; IR (KBr) ν_{max}: 3399, 2872, 1676, 1527, 1484, 1391, 1308, 1119, 828 cm^{-1}; ^1H NMR (MeOD): δ 1.10 (t, *J*=7.2 Hz, 3H), 1.76 (q, *J*=7.3 Hz, 2H), 3.04 (t, *J*=7.2 Hz, 2H), 3.11 (t, *J*=4.7 Hz, 2H), 3.61–3.77 (m, 10H), 7.65–7.75 (m, 2H), 7.76 (s, 1H), 7.94 (s, 1H); ^{13}C NMR (MeOD): δ 14.0, 22.7, 34.1, 40.6, 40.7, 68.0, 70.8, 71.4, 71.5, 114.5, 122.9, 126.7, 129.1, 133.0, 134.8, 145.4, 149.9, 150.6, 166.3; ^{19}F NMR (DMSO-d_6): δ −73.09 (s); HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{19}H_{27}BrN_3O_3S$: 456.0957, found 456.0959.

*4.1.7. Synthesis of 2-(2-(2-(2-(2-aminoethoxy)ethoxy) ethylcarbamoyl)-6-bromoquinolin-4-yl)thiobenzoic acid (**8e**).* To a stirred solution of thiosalicylic acid (154 mg, 1.0 mmol) in dry DMF (5 mL) at 0 °C was slowly added sodium hydride (72 mg, 3.0 mmol). The resulting suspension was warmed to room temperature and stirred for 1 h. *N*-(2-(2-(2-Aminoethoxy)ethoxy)-ethyl)-6-bromo-4-chloroquin-oline-2-carboxamide **7** (208 mg, 0.499 mmol) in dry DMF (2 mL) was then added and the mixture was stirred for 24 h at room temperature. The reaction was slowly quenched with water (50 mL) and extracted with ethyl acetate (2×50 mL) to remove excess thiosalicylic acid. The aqueous extract was concentrated in vacuo and acidified with ethanolic hydrochloric acid (20 mL) and then evaporated to dryness. The residue was purified by flash chromatography (MeOH/EtOAc, 1:1 with 1% Et_3N) to give 2-(2-(2-(2-(2-aminoethoxy)ethoxy)-ethylcarbamoyl)-6-bromo-quinolin-4-yl)thio)benzoic acid (128 mg, 48%) as a brown liquid; IR (KBr) ν_{max}: 3387, 2928, 1691, 1593, 1545, 1486, 1421, 1397, 1293, 1183, 1130, 1012, 824, 763 cm^{-1}; ^1H NMR(DMSO-d_6): δ 2.76 (t, *J*=5.3 Hz, 2H), 3.45–3.58 (m, 10H), 7.12 (dd, *J*=10.3, 1.4 Hz, 1H), 7.27 (dt, *J*=10.3, 2.2 Hz, 1H), 7.37 (dt, *J*=10.0, 1.8 Hz, 1H), 7.77 (s, 1H), 7.78 (dd, *J*=10.0, 2.1 Hz, 1H), 7.94 (dd, *J*=9.0, 2.1 Hz, 1H), 8.02 (d, *J*=9.0 Hz, 1H), 8.29 (d, *J*=2.7 Hz, 1H), 8.80(t, *J*=7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6): δ 38.4, 38.8, 66.7, 68.6, 69.5(×2), 119.4, 121.7, 126.0, 128.1, 128.4, 129.1, 129.5, 129.6, 132.1, 132.5, 133.9, 142.2, 144.5, 148.1, 149.6, 163.4, 169.7; ^1H NMR(MeOD/DMSO-d_6): δ 2.76 (t, *J*=5.1 Hz, 2H), 3.45–3.60 (m, 10H), 7.26–7.30 (m, 2H), 7.34–7.40 (m, 1H), 7.60 (d, *J*=8.1 Hz, 1H), 7.61 (s, 1H), 7.78 (dd, *J*=9.0, 2.1 Hz, 1H), 7.90 (d, *J*=9.0 Hz, 1H), 8.31 (d, *J*=2.1 Hz, 1H); ^{13}C NMR (MeOD/DMSO-d_6): δ 40.4, 40.8, 68.3, 70.8, 71.4, 71.5, 119.1, 123.4, 127.6, 127.9, 129.8, 130.0, 130.7(×2), 133.4, 135.3, 136.1, 146.3, 150.9, 151.2, 166.4, 168.2; HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{25}BrN_3O_5S$: 534.0698, found 534.0694.

*4.1.8. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(4-hydroxyphenylthio)quinoline-2-carboxamide trifluoroacetate salt (**8f**).* To a stirred solution of 4-hydroxy thiophenol (63 mg, 0.50 mmol) in dry methanol (5 mL) was slowly added sodium methoxide (0.50 mL, 25% w/w). The resulting suspension was stirred for 1 h at room temperature and then *N*-(2-(2-(2-amino-ethoxy)ethoxy)ethyl)-6-bromo-4-chloroquin-oline-2-carbox-amide **7** (105 mg, 0.252 m mol) in dry methanol (2 mL) was added. The mixture was stirred for 24 h at room temperature and the reaction was then quenched with water (50 mL), concentrated in vacuo and the residue extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by reverse phase flash chromatography (acetonitrile/water, 1:1 with 1% TFA) to give *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-bromo-4-(4-hydroxy-phenyl- thio) quinoline-2-carboxamide (87 mg, 57%, as the TFA salt)[12] as a yellow solid. MP: 133.8–135.2oC; IR (KBr) ν_{max}: 3088 (br), 2929, 1675, 1599, 1581, 1534, 1484, 1431, 1282, 1203, 1132, 834, 722 cm^{-1}; ^1H NMR (MeOD): δ 3.06 (t, *J*=5.1 Hz, 2H), 3.59 (t, *J*=5.0 Hz, 2H), 3.64–3.69 (m, 8H), 6.98 (d, *J*=8.8 Hz, 2H), 7.46 (d, *J*=8.8 Hz, 2H), 7.50 (s, 1H), 7.88 (dd, *J*=9.0, 2.1 Hz, 1H), 7.96 (d, *J*=9.0 Hz, 1H), 8.34 (d, *J*=2.1 Hz, 1H); ^{13}C NMR (MeOD): δ 40.5, 40.8, 68.0, 70.8, 71.5(×2), 115.8, 117.4, 118.8(×2), 123.3, 126.8, 128.7, 133.2, 135.3, 139.1(×2), 146.0, 150.6, 153.5, 161.5, 166.5; ^{19}F NMR (DMSO-d_6): δ −73.16 (s); HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{25}BrN_3O_4S$: 506.0749, found 506.0740.

*4.1.9. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(2-(tert-butylamino)ethylthio) quinoline-2-carboxamide (**8g**).* To a stirred solution of BOC-cystamine (88.5 mg, 0.50 mmol) in dry THF (20 mL) at 0 °C was slowly added 60% sodium hydride emulsion (70 mg, 1.75 mmol). The resulting suspension was warmed to room temperature and then stirred for 20 min. *N*-(2-(2-(2-Aminoethoxy) ethoxy)ethyl)-6-bromo-4-chloroquinoline-2-carboxamide **7** (105 mg, 0.252 m mol) in dry THF (2 mL) was then added and the mixture was stirred for 4 h at room temperature. The reaction was then quenched with water (50 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography (MeOH/EtOAc, 1:1 with 1% Et_3N) as eluent to give *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-bromo-4-(2(*tert*-butyl-amino)ethyl-thio)quinoline-2-carboxamide (133 mg, 95%) as an off-white solid. MP: 83.5–84.8 °C; IR (KBr) ν_{max}: 3312, 2865, 1717, 1655, 1529, 1482, 1171, 1132, 840 cm^{-1}; ^1H NMR (MeOD): δ 1.41 (s, 9H), 2.80 (t, *J*=5.2 Hz, 2H), 3.31(t, *J*=6.5 Hz, 2H), 3.45 (t, *J*=6.5 Hz, 2H), 3.54 (t, *J*=5.3 Hz, 2H), 3.64–3.76 (m, 8H), 7.77 (dd, 1H, *J*=9.0, 2.0 Hz, 1H), 7.84 (d, *J*=9.0 Hz, 1H), 7.93 (s, 1H), 8.12 (d, *J*=1.8 Hz, 1H); ^{13}C NMR (MeOD): δ 28.9(×3), 32.4, 40.1, 40.7, 42.1, 70.8, 71.5, 71.6, 73.0, 80.4, 115.1, 123.2, 127.0, 129.5, 133.2, 135.1, 145.8, 149.8, 150.2, 158.4, 166.4; HRMS (ES): *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{34}BrN_4O_5S$: 557.1433, found 557.1426.

*4.1.10. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-(iso-propylthio)quinoline-2-carboxamide trifluoroacetate salt (**8h**).* To a stirred solution of isopropanethiol (38 mg, 0.50 mmol) in dry methanol (5 mL) was slowly added sodium methoxide (0.5 mL, 25% w/w). The resulting suspension was stirred for 1 h at room temperature and then *N*-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-chloroquinoline-2-carboxamide **7** (105 mg, 0.252 mmol) in dry methanol (2 mL) was added. The mixture was stirred for 24 h at room temperature and the reaction then quenched with water (50 mL), concentrated in vacuo, and the residue extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by reverse phase flash chromatography (acetonitrile/water, 0–80% gradient elution with 1% TFA) to give *N*-(2-(2-(2-aminoethoxy)-ethoxy)ethyl)-6-bromo-4-(*iso*-propylthio)quinoline-2-carbox-amide (78 mg, 55%, as the TFA salt)[12] as a brown semi-solid; IR (KBr) ν_{max}: 3394, 2929, 1677, 1533, 1484, 1204, 1132, 834, 780, 722 cm^{-1}; ^1H NMR(MeOD): δ 1.49 (d, *J*=6.6 Hz, 6H), 3.10 (t, *J*=5.1 Hz, 2H), 3.68–3.78 (m, 10H), 3.87 (dq, *J*=6.7, 6.7 Hz, 1H), 7.85 (d, *J*=9.1 Hz, 1H), 7.94 (d, *J*=9.1 Hz, 1H), 8.08 (s, 1H), 8.26 (s, 1H); ^{13}C NMR (MeOD): δ 23.1(×2), 37.4, 40.6, 40.8, 68.0, 70.8, 71.5(×2), 116.4,

123.2, 127.3, 129.9, 133.4, 135.2, 146.2, 150.1, 150.3, 166.7; ^{19}F NMR (DMSO-d_6): δ −73.08 (s); HRMS (ES): m/z $[M+H]^+$ calcd for $C_{19}H_{27}BrN_3O_3S$: 456.0957, found 456.0956.

*4.1.11. Synthesis of 4-(2-(2-(2-(2-aminoethoxy)ethoxy) ethylcarbamoyl)-6-bromoquinolin-4-ylthiobenzoic acid (**8i**).* To a stirred solution of 4-mercaptobenzoic acid (28 mg, 0.180 mmol) in dry DMF (4 mL) was slowly added sodium hydride (13 mg, 0.542 mmol). The resulting suspension was stirred for 1 h at room temperature and then *N*-(2-(2-(2-aminoethoxy)ethoxy ethyl)-6-bromo-4-chloroquinoline-2-carboxamide **7** (50 mg, 0.120 mmol) in dry DMF (2 mL) was added. The mixture was stirred for 2 h at room temperature and the reaction then quenched with water, evaporated to dryness, and purified by flash column chromatography (MeOH/EtOAc, 1:1 with 1% Et_3N) to give 4-(2-(2-(2-(2-aminoethoxy)ethoxy)ethylcarbamoyl)-6-bromo-quinol-in-4-ylthio)benzoic acid (59 mg, 92%) as an off-white solid; IR (KBr) ν_{max}: 3433, 2999, 1691, 1593, 1545, 1420, 1293, 1184, 1116, 824, 764 cm^{-1}; ^{1}H NMR (MeOD/$CDCl_3$): δ 3.09 (t, J=4.8 Hz, 2H), 3.62 (t, J=4.9 Hz, 2H), 3.67–3.73 (m, 8H), 7.61 (d, J=8.4 Hz, 2H), 7.84 (s, 1H), 7.92 (dd, J=9.0, 1.9 Hz, 1H), 8.04 (d, J=9.0 Hz, 1H), 8.09 (d, J=8.4 Hz, 2H), 8.43 (d, J=1.9 Hz, 1H); ^{13}C NMR (DMSO-d_6): δ 38.3, 38.8, 66.5, 68.6, 69.3, 69.6, 117.4, 122.1, 125.5, 127.5, 130.9(×2), 131.8, 132.3, 133.8(×2), 134.4, 134.5, 144.5, 146.7, 149.6, 163.1, 166.4; HRMS (ES): m/z $[M+H]^+$ calcd for $C_{23}H_{25}BrN_3O_5S$: 534.0698, found 534.0689.

*4.1.12. Synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)-ethyl)-6-bromo-4-hydroxyquinoline-2-carboxamide (**8j**).* To a stirred solution of methyl 6-bromo-4-hydroxyquinoline-2-carboxylate **4** (100 mg, 0.355 mmol) in dry methanol (5 mL) were added 2,2′-ethylene dioxy-bis-ethylamine (148 mg, 1.0 mmol) and a catalytic amount of *p*-toluenesulfonic acid (10 mg). The resulting solution was stirred at room temperature for 24 h diluted with saturated $NaHCO_3$ (50 mL), concentrated in vacuo and the residue extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with brine (2×50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash chromatography (MeOH/EtOAc, 1:1 with 1% Et_3N) to give *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-6-bromo-4-hydroxyquin-oline-2-carboxamide (91 mg, 64%) as an off-white solid. MP: 229.2–230.6oC; IR (KBr) ν_{max}: 3233, 2928, 1676, 1626, 1597, 1509, 1203, 1130, 829, 722 cm^{-1}; ^{1}H NMR (MeOD): δ 3.12 (t, J=5.1 Hz, 2H), 3.63 (t, J=5.1 Hz, 2H), 3.68–3.73 (m, 8H), 6.89 (s, 1H), 7.76 (d, J=8.9 Hz, 1H), 7.81 (dd, J=8.9, 2.1 Hz, 1H), 8.31 (d, J=2.0 Hz, 1H); ^{13}C NMR (D_2O): δ 38.6, 39.2, 65.8, 68.0, 69.0, 69.1, 106.2, 117.6, 120.5, 124.7, 125.7, 135.4, 137.1, 141.2, 161.8, 177.6; HRMS (ES): m/z $[M+H]^+$ calcd for $C_{16}H_{21}BrN_3O_4$: 398.0715, found 398.0714.

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12. The amount of TFA present as a counter ion was quantified by ^{19}F (^{1}H decoupled) NMR experiment using spiked sample of trifluoroethanol as reference and found to contain 1:1 ratio of TFA as salt. As a consequence, on a weight basis the lowest effective concentrations that the active compounds **8b**, **8c**, **8d**, and **8h** were assayed as free quinoline compounds were ~20% lower than 80 nM, i.e., 67 nM.