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Hybrid Antibacterials. DNA polymerase:topoisomerase inhibitors

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Abstract

Novel Gram-positive (Gram+) antibacterial compounds consisting of a DNA polymerase IIIC (pol IIIC) inhibitor covalently connected to a topoisomerase/gyrase inhibitor are described. Specifically, 3-substituted-6-(3-ethyl-4-methylanilino)uracils (EMAUs) in which the 3-substituent is a fluoroquinolone moiety (FQ) connected by various linkers were synthesized. The resulting "AU-FQ" hybrid compounds were significantly more potent than the parent EMAU compounds as inhibitors of pol IIIC, and were up to 64-fold more potent as antibacterials *in vitro* against Gram+ bacteria. The hybrids inhibited the FQ targets, topoisomerase IV and gyrase, with potencies similar to norfloxacin, but tenfold lower than newer agents, e.g. ciprofloxacin and sparfloxacin. Representative hybrids protected mice from lethal *S. aureus* infection after intravenous dosing, and one compound showed protective effect against several antibiotic sensitive and resistant Gram+ infections in mice. The AU-FQ hybrids are a promising new family of antibacterials for treatment of antibiotic-resistant Gram+ infections.

Introduction

The emergence of antibiotic-resistant Gram+ bacterial infections, notably with *Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium* and *Streptococcus pneumoniae*, has prompted development of new chemotherapeutic agents that selectively attack new bacterial targets. One new target which has been validated recently in Gram+ organisms is DNA polymerase IIIC (pol IIIC), a DNA-dependent DNA polymerase which is specifically required for replicative DNA synthesis in these organisms. Interference with pol IIIC function prevents the replication of the Gram+ host chromosome, thus killing the host.[1,2]

We previously reported that optimally substituted 3-substituted 6-anilinouracils, specifically derivatives of 6-(3-ethyl-4-methylanilino)uracil "EMAU", were potent inhibitors of DNA polymerase IIIC from the Gram+ bacterium *Bacillus subtilis*, and had potent antibacterial activity against a panel of Gram+ organisms.[3,4] Several 3-substituted EMAU derivatives such as HB-EMAU (1; see structure) showed a protective effect given intraperitoneally to *S. aureus* infected mice, and one derivative was active given subcutaneously to *S. aureus* infected

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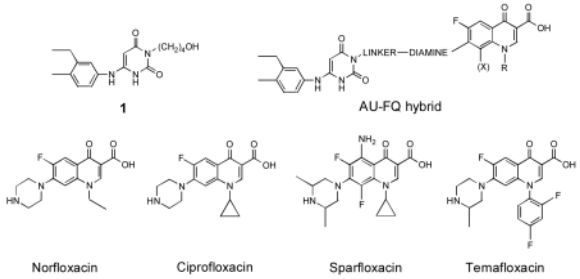
mice.[4] A related compound developed by another group was reported to have efficacy after intravenous dosing in the same animal model.[5]

Not all derivatives of EMAU that were potent enzyme inhibitors had significant antibacterial activity.[3] Factors that may limit antibacterial activity could include lack of penetration of the cell wall or membrane, removal of compound by active efflux mechanisms, and alteration of the sensitivity of the target enzyme in its "biophase" in the bacterium. In order to further explore the space available at the 3 position of EMAU and, thus, maximize binding to the pol IIIC target, we undertook additional synthesis in this class of compounds. Specifically, we have prepared derivatives of EMAU containing a variety of fluoroquinolones of known antibacterial activity (see typical structures) linked via their secondary amino groups, which we call "AU-FQ hybrids" (see structure). Figure 1 summarizes the structures and considerable documented structure-activity relationships regarding efficacy[6,7] and toxicity[8] of the fluoroquinolones. Based on this information we chose substituents known to impart high antibacterial potency and low incidence of side effects to the parent "FQs" for coupling with EMAU and related pol IIIC inhibitors. We report that these hybrid compounds have high potency against normal and antibiotic-resistant Gram+ bacteria in culture and against relevant infections in mice, and that they inhibit both bacterial targets, DNA polymerase IIIC and topoisomerase/gyrase.



Chemistry

Scheme 1 illustrates the multiple approaches available to synthesize AU-FQ hybrid compounds. The simplest approach utilized pre-existing 3-(iodoalkyl)EMAUs and 7-piperazinylfluoroquinolones (Scheme 2). Direct reaction between IB-EMAU and norfloxacin or ciprofloxacin was an obvious choice, but we were concerned that contamination of the product with even a small amount of the potent FQ itself could compromise the antibacterial results. Therefore, we compared the properties of compound 2 synthesized both by direct coupling between IB-EMAU and norfloxacin in DMF, and by treatment of the allyl ester of norfloxacin with IB-EMAU in DMF, followed by purification of the intermediate AU-FQ ester, and hydrolysis (LiOH). In both cases 2 was obtained in good yields, and the inhibitory properties were identical (data not shown). However, for all subsequent syntheses, esters of the FQs were used for reaction with iodoalkyl-EMAUs, followed by hydrolysis, to afford the hybrids in Scheme 2 in good yields. Compounds with characteristic small alkyl groups (Et, cPr, t-Bu) and fluorophenyl groups at the 1 position and various substituents at position 8 (halo, methoxy) were made in this way. However, in FQs halogenation of the 8-position can result in compounds with severe phototoxicity, and fluorophenyl groups at the 1-position can be antigenic.[9] In another strategy, 3-(4-piperazinylbutyl)EMAU reacted directly with a 6,7,8-trifluoroquinolone ester, yielding compound 4 (Scheme 3). A third strategy required displacement of the 7-fluoro group of polyfluoroquinolone esters by substituted piperazines, followed by reaction with 3-(iodobutyl)EMAU and hydrolysis (Scheme 4). In this manner, several 3-substituted-piperazinyl AU-FQ hybrids and their enantiomers were prepared. Several derivatives bearing bicyclic diamine substituents in the FQ portion were prepared via the same

strategy (Scheme 5). Some potent antibacterial fluoroquinolones, e.g. trovafloxacin, contain bicyclic diamines at position 7.[6,7]

Hybrids with linkers connecting the EMAU and 7-piperazinylfluoroquinolone portions other than butyl, including those with pentyl, heptyl and ethoxyethyl groups, were prepared by methods analogous to the above. Except for the pentyl derivatives, e.g. 6, none was comparable in activity to those described in Table 1 (results not shown).

Results

3-Substituted-EMAU derivatives where the 3-substituent was a "FQ" were potent inhibitors of pol IIIC. The results presented in Table 1 show the potent inhibition (K_i range = 0.004–0.04 μM) of *B. subtilis* pol $IIIC_{10}$ by all EMAU-FQ compounds. Significantly, an analog based on the less pol IIIC-inhibitory 3,4-dimethylanilino compound "DMAU" (18) retained good activity against pol IIIC (K_i = 0.1 μM), but a derivative bearing the essentially inactive 6-anilino group (19) was much less potent (K_i = 29 μM). The AU-FQ hybrid compounds had significantly lower MIC values against the screening set of Gram+ bacteria than typical EMAU derivatives, such as HB-EMAU (1) (Table 1). MIC values for AU-FQ compounds were ca. 2–4-fold lower than those for 1, although not as potent as the FQ standards norfloxacin, ciprofloxacin, and sparfloxacin against the corresponding FQ-sensitive organisms. Among the piperazinyl and substitutedpiperazinyl derivatives, most had similar potencies against individual strains, varying about ±2-fold in MIC values. Derivatives with bicyclic diamines (15–17) were generally less active, and had variable and weak activity against MRSA 1094 and the enterococci. The hybrid derived from DMAU (18) was nearly as active as its EMAU counterpart 5, but the unsubstituted 6- anilinouracil hybrid 19 was less potent, especially against the MRSA and enterococcal strains (Table 1).

The Gram+ antibacterial potency of the AU-FQ compounds was similar to that of the recently approved drug linezolid (Table 1). As with linezolid, the strains that were relatively resistant to FQs, e.g. MRSA 1094, *E. faecium* 19434 and VRE 700802, remained highly sensitive to the hybrids. Hybrids containing the 3-methylpiperazinyl moiety (12 and 13) were marginally more potent than other hybrids, consistent with the higher potency of the corresponding FQs compared with ciprofloxacin.[11] In the case of 13, the R enantiomer was slightly, but probably not significantly, more potent (Table 1). The enantiomers of the related 3-methylpiperazinyl FQ temafloxacin (see structure) showed little difference in MIC vs a large panel of Gram+ and Gram- bacteria, although the S isomer was 2–4-fold more potent in mouse protection tests than the R isomer.[11]

The Gram- organism *E. coli* was resistant to most compounds at the highest concentrations tested, although several compounds showed moderate activity (Table 1), consistent with the probability that these compounds may inhibit topo/gyrase in Gram- bacteria. No AU compounds, e.g. 1 (Table 1), show activity against Gram- bacteria, because they lack the Gram +-specific target, pol IIIC.[3]

The effect of the hybrid compounds on the fluoroquinolone targets topoisomerase IV (topo) and gyrase targets was tested by assaying several hybrid compounds and FQ standards against the enzymes isolated from *B. subtilis*.[12] Compounds 5, 13 and its enantiomers 13S and 13R, inhibited the decatenation reaction by topo and gyrase with potencies similar to or better than those of nalidixic acid, the prototype of the topoisomerase inhibitors, but considerably less than the effective drugs ciprofloxacin and sparfloxacin (Table 2). That the hybrid compounds exert action on both targets in sensitive bacteria has been demonstrated by experiments with *S. aureus* mutants resistant to one or both AU and FQ components.[13] For example, *S. aureus* strains resistant to HB-EMAU or to ciprofloxacin were fully sensitive to compound 13, but a

doubly-resistant mutant was insensitive to 13. Further details of target potencies and mechanism of action of the representative hybrid 13 are the subjects of a separate manuscript submitted for publication.[13]

Studies in mice

Compound 5, which is effectively the combination of HB-EMAU and ciprofloxacin, was selected as a representative compound with which to explore the properties of the AU-FQ hybrids *in vivo*. At acidic or alkaline pH, 5 and other hybrids were only slightly soluble in physiological saline. To achieve concentrations required for pharmacokinetic analysis and *in vivo* antibacterial testing at an acceptable dose volume, a solubility of 10–20 mg/ml was required. As a result, the solubility of 5 in saline was tested in the presence of several cosolvents. Solubility of 20 mg/ml was achieved with a formulation consisting of 10% N,N-dimethylacetamide (DMA) and 10% Cremophor EL in phosphate buffered saline, adjusted to pH 9.5. This "DCP" vehicle was used as vehicle for intravenous (iv) administration of 5 and related compounds via the tail vein in mice.

Pharmacokinetics of 5

Disposition studies were carried out with 5 dissolved in DCP via tail vein injection to mice at 100 mg/kg. (Higher doses caused agitation and discoloration of the tail.) Mice were dosed and blood was collected from groups of 3 animals at various times post- injection for quantitation by HPLC (see Experimental Section for details). Figure 2 shows the resulting plasma concentrations of 5. Peak concentration was 213 μg/mL at 5 minutes, and the concentration declined biexponentially thereafter. Non-compartmental analysis of the data by WinNonlin software gave an elimination half-life of 46 minutes. The mean MIC of 5 for the organism used in antibacterial screening in mice, *S. aureus* (Smith), is 0.41 ±0.15 μg/mL. Therefore, the plasma concentration of 5 was expected to exceed MIC for at least six hours following a iv dose of 100 mg/kg; the plasma concentration at 6 hours was 0.62 ±0.22 μg/mL.

HPLC analysis of the plasma samples from the above 5-treated mice revealed a faster eluting peak whose concentration was time-dependent. The plasma profile of this putative metabolite in mouse plasma is shown in Figure 2. This peak was isolated and subjected to LC-MS analysis. The observed M^{-1} = 806 was consistent with a glucuronide of 5. The elimination half-life of the metabolite was 57 minutes, and the estimated AUC(0−t) ratio (metabolite/5) was 3.7. The appearance of a glucuronide of 5 is consistent with the formation of carboxyl glucuronides of several fluoroquinolones,[14] although not of ciprofloxacin itself, which is excreted largely unchanged in human patients.[15] No metabolite that could represent the breakage of the EMAU and FQ components was observed under these conditions.

Acute toxicity of 5 in mice

Intravenous doses of 5 in DCP via the tail vein were well tolerated by mice (n = 5) at 100 mg/kg (dose volume of 5 mL/kg), both as a single dose or as two 50 mg/kg doses separated by 1 hour. At 200 mg/kg the mice showed a transient, seizure-like reaction to a single injection after ca. 10 minutes, but all animals appeared normal after 16 hours.

Efficacy of 5 in the *S. aureus* (Smith) ip infection model

Using pharmacokinetic and modeling data, a series of experiments was designed to test the effect of 5 in the standard murine peritonitis model of *S. aureus* following iv injection. The objective was to determine antibacterial efficacy *in vivo* from a site distinct from the infection site. Swiss-Webster mice were infected intraperitoneally (ip) with 10^8 colony forming units (CFU) of *S. aureus* (Smith), and survival was monitored for 72 hours. Typically animals die within 10–18 hours of the infection, and the positive control drug vancomycin, given ip at 10

mg/kg or iv at 30 mg/kg, protects all animals for at least 72 hours. Compound 5 was partially effective in protecting mice from ip *S. aureus* (Smith) infection when administered in a single dose of 50 mg/kg and completely effective at 75 and 100 mg/kg (Table 3). Vancomycin as positive control was fully protective at 30 mg/kg.

Comparison of iv efficacy of AU-FQ compounds against *S. aureus* (Smith) infections

Representative hybrid compounds were screened, by iv dosing in DCP, for protective activity against *S. aureus* (Smith) infection in mice. Single doses of all hybrids protected animals for 72 hours, and their ED_{50} values are summarized in Table 4. Compound 13 was somewhat more potent than 5, and its enantiomers 13R and 13S were among the most potent hybrids in these experiments.

Spectrum of *in vivo* activity of 13

The significant potency of compound 13 in the *S. aureus* infection model in mice prompted detailed study of this compound against infections caused by additional bacterial strains. Ip infections in mice were established with *E. faecalis* and the drug resistant isolates MRSA 1094 and VRE 700802. However, these organisms are less virulent in mice than *S. aureus*, and required higher CFUs and 5% mucin as adjuvant to cause lethal infections. Even then, the mean survival times from these infections were longer than for *S. aureus* (Smith), proving a challenge for bolus iv dosing with AU-FQ compounds. The results of Table 5, based on cumulative results for several experiments, show that single iv doses of 13 had little protective effect, except for E. faecalis where 80% protection was seen at 75 mg/kg. Double doses of 75 mg/kg, separated by 120 minutes, gave statistically significant, although not complete, protection from MRSA and VRE infections. In the VRE infection, significant increases in mean survival times were noted from single and double doses of 13 (data not shown).

Discussion

The properties of AU-FQ hybrid compounds described in this paper and its companion paper[12] show that these are unique, dual action antibacterials with potential for treatment of antibiotic-resistant, Gram+ bacterial infections. The SAR for active hybrid compounds is relatively “flat”, i.e. there is little variation in antibacterial potency against individual strains except for those compounds containing AU substituents other than “3-ethyl-4-methyl”, compounds with longer linkers, or bicyclic diamines. There have been several reports of hybrid antibiotics consisting of fluoroquinolones covalently connected to penicillins, cephalosporins and carbapenems;[16] the linkages in these compounds may cleave, hydrolytically or enzymatically, to give the individual components. The limited observations for hybrid 5 in mice suggest that glucuronidation is a major pathway of metabolism. Cleavage to the respective pol IIIC and topoisomerase/gyrase inhibitor moieties, although not observed, cannot be ruled out, however.

The AU-FQ hybrids were fully protective in protecting mice from lethal infection by *S. aureus* (Smith) at nontoxic doses (Tables 3 and 4), and a representative compound (13) gave statistically significant protection of mice with lethal infections by other Gram+ bacteria (Table 5). The significant antibacterial activity of 13, both *in vitro* and *in vivo*, have warranted further study of this compound. A major challenge is its low water solubility, consistent with both the high molecular weight and likely zwitterionic property of the compound. The preparation and study of acid and base salts of 13 are underway. In addition, potential differences in the disposition and *in vivo* potencies of the enantiomers 13R and 13S have prompted more extensive comparisons of them with the racemate.

Experimental Section

Materials

Reagent chemicals, solvents and chromatographic media were obtained from commercial sources. 3-(4-Iodobutyl)-6-anilinouracils, 3-(4-piperazinylbutyl)EMAU, and 1 were synthesized as described.[4] 2-(Hydroxymethyl)piperazine was synthesized by a literature method.[17] Norfloxacin and ciprofloxacin hydrochloride were obtained from Sigma and Mediatech Inc., respectively. Esters of polyhaloquinolone-3-carboxylic acids were prepared by methods cited individually below.

Analytical HPLC was performed with a Hitachi LC using a Waters Symmetry C8 column (3.5 μm, 4.6 × 50 mm) and an elution gradient from 0 to 50% MeCN:H_2O in 20 min (unless indicated otherwise). Preparative HPLC was performed on a Waters Delta Prep 400 system using a Waters Symmetry Prep C8 column (7 μm, 19 × 150 mm) and a gradient from 25 to 50% MeCN:H_2O. Plasma samples were analyzed with a Varian Prostar System using a Microsorb C18 column (5 m, 4.6 × 150 mm), a mobile phase of MeCN:H_2O:NEt_3:AcOH (25:74.7:0.2:0.1), and a detection wavelength of 282 nm, as described previously.[4]

Melting points were determined on a Mel-temp apparatus and are uncorrected. Unless otherwise noted, NMR spectra were obtained in Me_2SO-d_6 solution with a Bruker Avance 300 or a Varian INOVA 400 spectrometer. Chemical shifts (δ) are in ppm from internal TMS. Mass spectra were measured on a ThermoFinnigan LCQ Advantage ion trap instrument by APC ionization. All new compounds were characterized for identity by HR-MS with a FAB source on a Kratos MS50TCTA spectrometer equipped with a peak matching unit, or by EI on a JEOL JMS-700 MStation double focusing sector spectrometer (Mass Spectrometry Facility, University of Massachusetts, Amherst). All new compounds were at least 98% by reverse phase HPLC in two solvent systems, except 15–17 for which one solvent system was used (Supporting Information).

3-{4-[1-(1-Ethyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 2

Method a—A mixture of 3-(4-iodobutyl)-6-(3-ethyl-4-methylanilino)uracil (IB-EMAU) (241 mg, 0.56 mmol), K_2CO_3 (130 mg, 0.94 mmol), and norfloxacin (150 mg, 0.47mmol) in DMF (20 ml) was stirred at room temperature overnight. The mixture was concentrated under reduced pressure, and the residue was purified by chromatography on silica gel using a gradient of MeOH in $CHCl_3$ as eluent to give 180 mg (62%) of product as a colorless solid. ^{1}H NMR: 15.31 (s, 1H, COOH), 10.37 (s, 1H, NH), 8.92 (s, 1H, FQ-C_2-H), 8.07 (s, 1H, NH), 7.91 (d, 1H, FQ-C_5-H), 6.90–7.23 (m, 4H, Ar-H and FQ-C_8-H), 4.71 (s, 1H, C_5-H), 4.57 (q, 2H, NCH_2), 3.72 (t, 2H, NCH_2), 3.30 (m, 4H, 2×CH_2N), 2.57 (m, 6H, 2×CH_2N and $ArCH_2$), 2.37 (m, 2H, CH_2N), 2.21 (s, 3H, $ArCH_3$), 1.37–1.58 (m, 7H, 2×CH_2 and CH_3), 1.12 (t, 3H, $ArCH_2CH_3$). HRMS: calcd for $C_{33}H_{40}FN_6O_5$ (M + 1) 619.2966; found 619.3083.

Method b—A mixture of allyl 1-ethyl-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate hydrochloride[18] (0.85 g, 2.1 mmol), $NaHCO_3$ (0.56 g, 6.7 mmol), and IB-EMAU (1.1 g, 2.6 mmol) in DMF (60 mL) was stirred at room temperature overnight. Water was added, and the mixture was extracted with $CHCl_3$, and the organic extracts were dried over Na_2SO_4. After removal of solvents, the residue was purified by chromatography on silica gel using 10–15% MeOH:$CHCl_3$ as eluent to give 877 mg of ester (62%) as a colorless solid. This ester (600 mg) was dissolved in 80 mL of a 4:1mixture of MeOH and water. Solid LiOH (53 mg) was added to the solution, and the mixture was stirred at room temperature overnight. The mixture was acidified with AcOH to pH 5–6. The solvent was evaporated to dryness, and a small amount

of water was added to the residue. The suspension was filtered and dried *in vacuo* to give 557 mg (99%) of product as an off-white solid, identical with the sample made by method a.

3-{4-[1-(1-Ethyl-3-carboxy-4-oxo-6-fluoro-8-aza-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 3

Ethyl 1-ethyl-6-fluoro-7-chloro-8-aza-4-quinolone-3-carboxylate[19] was treated with IB-EMAU and K_2CO_3 in DMF as above. Hydrolysis of the resulting ester with aqueous LiOH as above gave the product in 70% yield. ^{1}H NMR: 15.20 (s, 1H, COOH), 10.38 (s, 1H, NH), 8.86 (s, 1H, FQ-C_2-H), 8.06 (s, 2H), 6.85–7.14 (m, 3H, Ar-H), 4.72 (s, 1H, C_5-H), 4.55 (m, 2H, CH_2), 3.88 (m, 4H, 2 × NCH_2), 3.50 (m, 2H, CH_2N), 3.30 (m, 2H, CH_2N), 2.55 (q, 2H, CH_2), 2.34 (m, 4H, 2 × CH_2N), 2.15 (s, 3H, $ArCH_3$), 1.56 (m, 7H, 2 × CH_2 and CH_3), 1.10 (t, 3H, CH_3). HRMS: calcd. for $C_{32}H_{39}FN_7O_5$ 620.2996; found 620.2995.

3-{4-[1-(1-Ethyl-3-carboxy-4-oxo-6,8-difluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 4

A solution of 3-[4-(1-piperazinyl)butyl]-6-(3-ethyl-4-methylanilino)uracil dihydrochloride (1.2 eq), ethyl 1-ethyl-6,7,8-trifluoro-4-quinolone-3-carboxylate[20] (1 eq), and K_2CO_3 (4.0 eq) in MeCN was heated at reflux for 16 h. The solvent was evaporated, and the residue was chromatographed on silica gel with $CHCl_3$:MeOH as eluent, giving ca. 40% of the ester intermediate. The ester was stirred in a solution of LiOH in aqueous MeOH at room temperature overnight. After evaporation of MeOH, the solution was acidified with glacial AcOH. The colorless precipitate was filtered and washed with water. Yield: 90%. ^{1}H NMR: 14.82 (s, 1H, COOH), 10.40 (s, 1H, NH), 8.90 (s, 1H, FQ-C_2-H), 8.02 (s, 1H, NH), 7.89 (d, 1H, FQ-C_5-H), 6.85–7.14 (m, 3H, Ar-H), 4.72 (s, 1H, C_5-H), 4.61 (m, 2H, CH_2), 3.83 (m, 2H, NCH_2), 3.42 (m, 6H, 3 × CH_2N), 2.57 (q, 2H, CH_2), 2.33 (m, 4H, 2 × CH_2N), 2.17 (s, 3H, $ArCH_3$), 1.54 (m, 7H, 2 × CH_2 and CH_3), 1.10 (t, 3H, CH_3). HRMS: calcd. for $C_{33}H_{39}F_2N_6O_5$ 637.2950; found 637.2951.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 5

A mixture of ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate (100 mg, 0.28 mmol), $NaHCO_3$ (74 mg, 0.88 mmol), and IB-EMAU (184 mg, 0.43 mmol) in 30 mL of DMF was stirred at room temperature overnight. Water was added, and the mixture was extracted with $CHCl_3$. The organic extracts were dried over Na_2SO_4, and, after removal of solvents, the residue was purified by chromatography on silica gel using a gradient of 7–15% MeOH:$CHCl_3$ as eluent to give 139 mg (76%) of ester as a colorless solid. ^{1}H NMR: 10.42 (s, 1H, NH), 8.30 (s, 1H, FQ-C_2-H), 8.12 (s, 1H, NH), 7.78 (d, 1H, FQ-C_5-H), 7.44 (d, 1H, FQ-C_8-H), 6.90–7.15 (m, 3H, Ar-H), 4.75 (s, 1H, C_5-H), 4.20 (q, 2H, CH_2O), 3.72 (m, 2H, NCH_2), 3.65 (m, 1H, CH), 3.22 (m, 4H, 2 × CH_2N), 2.50–2.63 (m, 6H, 2 × CH_2N and $ArCH_2$), 2.35 (m, 2H, NCH_2), 2.21 (s, 3H, $ArCH_3$), 1.38–1.60 (m, 4H, 2 × CH_2), 1.20–1.30 (m, 5H, CH_2 and CH_3), 1.05–1.17 (m, 5H, CH_2 and $ArCH_2CH_3$). This ester (100 mg) was dissolved in 50 mL of 4:1 MeOH:H_2O. Solid LiOH (40 mg) was added to the solution, and the mixture was stirred at room temperature overnight. After acidification with glacial AcOH to pH 5–6, the solvent was evaporated to dryness, and a small amount of water was added. The suspension was filtered and the solid dried *in vacuo* to give 87 mg (91%) of product as an off-white solid. ^{1}H NMR: 15.18 (s, 1H, COOH), 10.37 (s, 1H, NH), 8.65 (s, 1H, FQ-C_2-H), 8.05 (s, 1H, NH), 7.90 (d, 1H, FQ-C_5-H), 7.56 (s, 1H, FQ-C_8-H), 6.90–7.17 (m, 3H, Ar-H), 4.71 (s, 1H, C_5-H), 3.83 (m, 1H, CH), 3.72 (m, 2H, NCH_2), 3.30 (m, 4H, 2 × CH_2N), 2.57 (m, 6H, 2 × CH_2N and $ArCH_2$), 2.37 (m, 2H, CH_2N), 2.21 (s, 3H, $ArCH_3$), 1.40–1.61 (m, 4H, 2 × CH_2), 1.32 (m, 2H, CH_2), 1.10–1.28 (m, 5H, CH_2 and CH_3). HRMS: calcd for $C_{34}H_{40}FN_6O_5$(M + 1) 631.2966; found 631.3029.

3-{5-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]pentyl}-6-(3-ethyl-4-methylanilino)uracil, 6

Ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate and 3-(5-iodopentyl)-6-(3-ethyl-4-methylanilino)uracil (IP-EMAU) were reacted as above, followed by LiOH hydrolysis of the ester intermediate. Yield: 66%. ^{1}H NMR (DMSO-d_6): 15.22 (s, 1H), 10.37 (s, 1H), 8.65 (s, 1H), 8.08 (s, 1H), 7.89 (d, 1H), 7.65 (d, 1H), 7.11 (d, 1H), 6.93 (m, 2H), 4.73 (s, 1H), 3.81 (m, 1H), 3.70 (t, 2H), 3.34 (m, 4H), 2.55–2.60 (m, 6H), 2.36 (t, 2H), 2.22 (s, 3H), 1.53 (m, 4H), 1.25–1.32 (m, 4H), 1.07–1.20 (m, 5H). HRMS: Calcd. for $C_{35}H_{42}FN_6O_5$ 645.3200. Found 645.3184.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-8-chloro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 7

Ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-piperazinyl-8-chloroquinoline-3-carboxylate[21] and IB-EMAU were reacted as above, followed by LiOH hydrolysis of the ester intermediate. Yield: 42%. ^{1}H NMR: 14.78 (s, 1H, COOH), 10.40 (s, 1H, NH), 8.90 (s, 1H, FQ-C_2-H), 8.04 (s, 1H), 7.88 (d, 1H), 6.85–7.15 (m, 3H), 4.72 (s, 1H), 4.12 (m, 1H), 3.68 (m, 2H), 3.30 (m, 4H), 2.58 (m, 6H), 2.36 (m, 2H), 2.18 (s, 3H), 1.40–1.61 (m, 4H), 0.95–1.26 (m, 7H). HRMS: calcd. for $C_{34}H_{39}ClFN_6O_5$ 665.2654; found 665.2662.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-8-methoxy-7-quinolyl)-4-piperazinyl] butyl}-6-(3-ethyl-4-methylanilino)uracil, 8

Ethyl 1-cyclopropyl-6-fluoro-8-methoxy-4-oxo-7-piperazinylquinoline-3-carboxylate[22] was converted to its BF_2 complex as described.[23] A mixture of this complex (59 mg, 144 mmol), IB-EMAU (74 mg, 0.173 mmol) and $NaHCO_3$ (36 mg, 0.429 mmol) in DMF (10 mL) was stirred at room temperature overnight. The mixture was concentrated under reduced pressure, and the residue was purified by chromatography on silica gel using a gradient of 5–10% MeOH:$CHCl_3$ as eluent to give the BF_2 complex intermediate. The intermediate was dissolved in 30 mL of 80% MeOH:H_2O, 1 mL trifluoroacetic acid was added, and the mixture was heated at reflux for 1.5 h. The pH was adjusted to 5–6 with 2N NaOH, the solvents were evaporated to dryness, and 30 mL of water was added. The suspension was filtered, and the solid was dried in vacuo to give 5.5 mg (38%) of product. ^{1}H NMR (DMSO-d_6): 14.92 (s, 1H), 10.35 (s, 1H), 8.71 (s, 1H), 8.13 (s, 1H), 7.74 (d, 1H), 7.15 (d, 2H), 6.93 (m, 2H), 4.73 (s, 1H), 4.10–4.30 (m, 1H), 3.78–3.86 (m, 4H), 3.30 (s, 2H), 2.60 (q, 2H), 2.23 (s, 3H), 1.30–1.70 (m, 4H), 0.90–1.30 (m, 4H). HRMS: calcd. for $C_{35}H_{42}FN_6O_6$ 661.3150; found 661.3135.

3-{4-[1-(1-t-Butyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil hydrochloride, 9

Ethyl 1-(t-butyl)-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate[24] and IB-EMAU were reacted as above, and the ester intermediate was hydrolyzed with aqueous LiOH. Yield: 63%. ^{1}H NMR (DMSO-d_6): 15.13 (s, 1H), 10.61 (s, 1H), 10.50 (s, 1H), 8.96 (s, 1H), 8.53 (s, 1H), 8.05 (d, 1H), 7.48 (d, 1H), 7.14 (d, 1H), 6.95 (m, 2H), 4.75 (s, 1H), 3.74–3.85 (m, 4H), 3.61 (m, 2H), 3.20–3.43 (m, 6H), 2.58 (q, 2H), 2.24 (s, 3H), 1.89 (s, 9H), 1.23 (m, 2H), 1.58 (m, 2H), 1.19 (t, 3H). HRMS: calcd. for $C_{35}H_{44}FN_6O_5$ 647.3357. Found 647.3343.

3-{4-[1-(1-{2,4-Difluorophenyl}-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 10

Ethyl 1-(2,4-difluorophenyl)-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate[25] and IB-EMAU gave the product after LiOH hydrolysis of the ester intermediate. Yield: 72%. ^{1}H NMR: 15.02 (s, 1H), 10.35 (s, 1H), 8.86 (s, 1H), 8.10 (s, 1H), 7.88 (m, 2H), 7.65 (t, 1H), 7.43 (t, 1H), 6.86–7.14 (m, 3H), 6.20 (d, 1H), 4.72 (s, 1H), 3.68 (t, 2H), 3.50 (m, 2H), 2.95 (m, 4H), 2.55

(q, 2H), 2.38 (m, 4H), 2.16 (s, 3H), 1.44 (m, 4H), 1.12 (t, 3H). HRMS. Calcd. for $C_{37}H_{38}F_3N_6O_5$ 703.2856; found 703.2861.

3-{4-[1-(1-(4-Fluorophenyl)-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3-ethyl-4-methylanilino)uracil, 11

Ethyl 1-(4-fluorophenyl)-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate[25] and IB-EMAU gave the product after LiOH hydrolysis of the ester intermediate. Yield: 78%. ^{1}H NMR (DMSO-d_6): 15.08 (s, 1H), 10.37 (s, 1H), 8.68 (s, 1H), 8.11 (s, 1H), 7.98 (d, 1H), 7.80 (m, 2H), 7.49 (m, 2H), 6.86–7.14 (m, 3H), 6.40 (d, 1H), 4.72 (s, 1H), 3.68 (t, 2H), 3.06 (m, 4H), 2.57 (m, 6H), 2.30 (m, 2H), 2.18 (s, 3H), 1.50 (m, 4H), 1.15 (t, 3H). HRMS: Calcd. for $C_{37}H_{39}F_2N_6O_5$ 685.2950; found 685.3004.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-(3-methylpiperazinyl)] butyl}-6-(3-ethyl-4-methylanilino)uracil, 12

A solution of ethyl 1-cyclopropyl-6,7-difluoro-4-oxo-quinoline-3-carboxylate[26] and 2-methylpiperazine in DMSO was heated at 80 °C for 3 h. The resulting ethyl 1-cyclopropyl-6,7-difluoro-4-oxo-7-(3-methylpiperazinyl)quinoline-3-carboxylate (isolated as described for the 8-fluoro analog[27]) was treated with IB-EMAU, followed by hydrolysis of the ester intermediate, to give the product. Yield: 38%. ^{1}H NMR: 15.10 (s, 1H), 10.37 (s, 1H), 8.78 (s, 1H), 8.05 (s, 1H), 7.88 (d, 1H), 6.88–7.17 (m, 3H), 4.72 (s, 1H), 3.83 (m, 1H), 3.70 (m, 2H), 3.50 (m, 4H), 2.30–3.30 (m, 10H), 2.18 (s, 3H), 1.10–1.65 (m, 14H). HRMS: calcd. for $C_{35}H_{42}FN_6O_5$ 546.3200; found 645.3196.

(R)-3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-(3-methylpiperazinyl)] butyl}-6-(3-ethyl-4-methylanilino)uracil, 12R

Treatment of ethyl 1-cyclopropyl-6,7-difluoro-4-oxoquinoline-3-carboxylate with (R)-2-methylpiperazine as above gave (R)-ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-(3-methylpiperazinyl)quinoline-3-carboxylate. Reaction with IB-EMAU and hydrolysis of the ester gave the (R) enantiomer. Yield: 65%. ^{1}H NMR: as for 12. HRMS: calcd. for $C_{35}H_{42}FN_6O_5$ 645.3200; found 645.3184.

(S)-3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-(3-methylpiperazinyl)] butyl} 6-(3-ethyl-4-methylanilino)uracil, 12S

Treatment of ethyl 6,7-difluoro-4-oxoquinoline-3-carboxylate with (S)-2-methylpiperazine gave (S)-ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-(3-methylpiperazinyl)quinoline-3-carboxylate. Reaction with IB-EMAU and hydrolysis of the ester gave the (S) enantiomer. Yield: 41%. ^{1}H NMR: as for 12. HRMS: calcd. for $C_{35}H_{42}FN_6O_5$ 645.3200; found 645.3198.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6,8-difluoro-7-quinolyl)-4-(3-methylpiperazinyl)] butyl}-6-(3-ethyl-4-methylanilino)uracil, 13

A mixture of ethyl 1-cyclopropyl-6,8-difluoro-4-oxo-7-(3-methylpiperazinyl)quinoline-3-carboxylate,[27] (320 mg, 0.82 mmol), $NaHCO_3$ (414 mg, 3 mmol), and IB-EMAU (580 mg, 1.36 mmol) in 50 mL DMF was stirred at room temperature overnight. Water was added, and the mixture was extracted with $CHCl_3$, and then dried over Na_2SO_4. After removal of solvents, the residue was purified by chromatography on silica gel using a gradient of 7–15% MeOH:$CHCl_3$ as eluent to give 196 mg (35%) of ester as a colorless solid. ^{1}H NMR: 10.48 (s, 1H), 8.52 (s, 1H), 8.05 (s, 1H), 7.78 (d, 1H), 6.86–7.15 (m, 3H), 4.78 (s, 1H), 4.17 (q, 2H), 3.90(m, 1H), 3.72 (m, 3H), 3.32 (m, 4H), 2.70–2.93 (m, 2H), 2.54 (q, 2H), 2.35 (m, 2H), 2.12 (s, 3H), 1.38–1.60 (m, 4H), 0.95–1.30 (m, 13H) ppm. The ester (85 mg, 0.12 mmol) was dissolved in 20% aqueous MeOH (30 mL), LiOH (40 mg, 0.95 mmol) was added, and the mixture was stirred at room temperature overnight. The mixture was acidified with AcOH to

pH 5–6, the solvent was evaporated to dryness, and a small amount of water was added to the residue. The suspension was filtered and dried *in vacuo* to give 72 mg (89%) of product as an off-white solid. ^{1}H NMR: 15.20 (s, 1H), 10.36 (s, 1H), 8.72 (s, 1H), 8.50 (s, 1H), 7.82 (d, 1H), 6.86–7.15 (m, 3H), 4.78 (s, 1H), 4.12 (m, 1H), 3.74 (m, 2H), 3.33 (m, 4H), 2.90 (m, 2H), 2.12 (s, 3H), 1.40–1.61 (m, 4H), 0.95–1.22 (m, 10H, CH_2). HRMS: calcd. for $C_{35}H_{41}F_2N_6O_5$ 663.3106; found 663.3163.

(R)-3-{4-[1-(1-cyclopropyl-3-carboxy-4-oxo-6,8-difluoro-7-quinolyl)-4-(3-methylpiperazinyl)] butyl}-6-(3-ethyl-4-methylanilino)uracil, 13R

This compound was prepared by the same procedure as for 13, but with the use of (R)-ethyl 1-cyclopropyl-6,8-difluoro-4-oxo-7-(3-methylpiperazinyl)quinoline-3-carboxylate. Yield: 45% overall. ^{1}H NMR: as for 13. HRMS: calcd. for $C_{35}H_{41}F_2N_6O_5$ 663.3106; found 663.3108.

(S)-3-{4-[1-(1-cyclopropyl-3-carboxy-4-oxo-6,8-difluoro-7-quinolyl)-4-(3-methylpiperazinyl)] butyl}-6-(3-ethyl-4-methylanilino)uracil, 13S

This compound was prepared by the same procedure as for 13, but with the use of (S)-ethyl 1-cyclopropyl-6,8-difluoro-4-oxo-7-(3-methylpiperazinyl)quinoline-3-carboxylate. Yield: 35% overall. ^{1}H NMR: as for 13. HRMS: calcd. for $C_{35}H_{41}F_2N_6O_5$ 663.3106; found 663.3136.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6,8-difluoro-7-quinolyl)-4-(3-hydroxymethylpiperazinyl)]butyl}-6-(3-ethyl-4-methylanilino)uracil, 14

A mixture of ethyl 1-cyclopropyl-6,8-difluoro-4-oxo-7-[3-(hydroxymethyl)piperazinyl] quinoline-3- carboxylate[28] (400 mg, 1 mmol), $NaHCO_3$ (250 mg, 3 mmol), and IB-EMAU (1.1g, 2.6 mmol) in DMF (80 mL) was stirred at room temperature overnight. The solvent was removed, and water was added to the residue. The suspension was extracted with $CHCl_3$, and the organic extracts were dried over Na_2SO_4. After removal of solvents, the residue was purified by chromatography on silica gel using 7–15% MeOH:$CHCl_3$ as eluent to give 320 mg (46%) of ethyl ester as a colorless solid. The ester (200 mg) was dissolved in 4:1MeOH:H_2O (80 mL), LiOH (60 mg) was added, and the solution was stirred at room temperature overnight. The solution was brought to pH 5–6 with glacial AcOH, and the solvents were evaporated to dryness, and a small amount of water was added to the residue. The suspension was filtered, and the solid was dried *in vacuo* to give 176 mg (92%) of product as an off-white solid. ^{1}H NMR: 15.20 (s, 1H), 10.45 (s, 1H), 8.68 (s, 1H), 8.42 (s, 1H), 7.8 (d, 1H), 6.86–7.14 (m, 3H), 4.76 (s, 1H), 4.57 (s, 1H), 4.08 (m, 1H), 3.74 (m, 4H), 2.3–3.6 (m, 11H), 2.18 (s, 3H), 1.38–1.6 (m, 4H), 1.05–1.26 (m, 7H). HRMS: calcd. for $C_{35}H_{41}F_2N_6O_6$ 679.3055; found 679.3046.

3-{5-[1-(1-Ethyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-3-(1,3-diazabicyclononyl)]pentyl}-6-(3-ethyl-4-methylanilino)uracil, 15

A mixture of 7-chloro-1-ethyl-6-fluoro-4-oxoquinoline-3-carboxylic acid[20] (270 mg, 1 mmol), 2,8-diazabicyclo[4.3.0]nonane trifluoroacetate (TFA)[29] (360 mg, 1 mmol,) and 1,4-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.45 ml, 3 mmol) in 1-methyl-2-pyrrolidinone (3 mL) was heated at 120 °C for 18 h. The solvent was removed, and the residue was purified by preparative HPLC with 25–50% MeCN:H_2O as eluent to obtain 91 mg (26 %) of intermediate. A mixture of this intermediate (50 mg, 0.14 mmol), IP-EMAU (60 mg, 0.14 mmol), and K_2CO_3 (60 mg, 0.43 mmol) in DMF (2 mL) was heated at 90 °C for 2 h. After removal of solvent, the residue was purified by preparative HPLC with 25–50% MeCN:H_2O as eluent to obtain 17 mg (10%) of 15 as the TFA salt. ^{1}H NMR: 10.94 (s, 1H), 10.34 (s, 1H), 9.59 (s, 1H), 9.32 (s, 1H), 8.66 (s, 1H), 8.32 (d, 1H), 7.56 (dd, 1H), 7.34–7.39 (m, 2H), 7.16 (m, 1H), 5.15 (s, 1H), 4.97 (m, 4H), 3.29–4.65 (m, 9H), 3.0 (q, 2H), 2.66 (s, 3H), 1.84–2.22 (m, 7H), 1.73 (m, 2H), 1.56 (t, 3H). HRMS: calcd for $C_{37}H_{46}FN_6O_5$, 673.3514; found, 673.3500.

3-{5-[1-(1-Cyclopropyl-3-carboxy-6-fluoro-4-oxo-7-quinolyl)-3-(1,3-diazabicyclononyl)] pentyl}-6-(3-ethyl-4-methylanilino)uracil, 16

A mixture of 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-quinoline-3-carboxylic acid[20] (360 mg, 1.28 mmol), 2,8-diazabicyclo[4.3.0] nonane[29] as the TFA salt (450 mg, 1.27 mmol), and DBU (0.6 ml, 3.84 mmol) in 1-methyl-2-pyrrolidinone (3 mL) was heated at 120 °C for 18 h. The solvent was removed, and the residue was purified by preparative HPLC as above to obtain 161 mg (34%) of intermediate as the TFA salt. A mixture of this intermediate (55.4 mg, 0.113 mmol), IP-EMAU (50 mg, 0.113 mmol), and K_2CO_3 (50 mg, 0.36 mmol) in DMF (2 mL) was heated at 90 °C for 1 h. After removal of solvent, the residue was purified by preparative HPLC as above to obtain 15.6 mg (20%) of 16 as the TFA salt. ^{1}H NMR: 11.06 (s, 1H), 10.46 (s, 1H), 9.72 (s, 1H), 9.16 (s, 1H), 8.77 (s, 1H), 8.41 (d,1H), 7.71 (m, 2H), 7.49 (m, 2H), 5.27 (s, 1H), 3.44–4.79 (m, 12H), 3.13 (q, 2H), 2.79 (s, 3H), 2.35 (m, 6H), 2.09 (m, 2H), 1.86 (m, 5H), 1.68 (m, 5H). HRMS: calcd for $C_{38}H_{46}FN_6O_5$, 685.3514; found, 685.3521.

3-{5-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-3-(5-oxa-1,3-diazabicyclononyl)]pentyl}-6-(3-ethyl-4-methylanilino)uracil, 17

A mixture of 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-quinoline-3-carboxylic acid[20] (0.25 g, 0.85 mmol), *cis*-2-oxa-5,8-diazabicyclo[4,3-0]nonane dihydrochloride[29] (0.18 g, 0.93 mmol), and DBU in DMF (5 mL) was heated at 95 °C for 5 h. The solvent was removed, and the residue was crystallized from MeOH:Et_2O to give 0.24 g (70%) of intermediate as a colorless solid. A mixture of the intermediate (0.1 g, 0.24 mmol), IP-EMAU (0.13 g, 0.29 mmol), and K_2CO_3 (51.6 mg, 0.37 mmol) in DMF (2 mL) was heated at 90 °C for 3 h. Water was added, the mixture was extracted with CH_2Cl_2, and the organic extracts were dried over Na_2SO_4. After removal of solvents, the residue was purified by chromatography on silica gel using a gradient of 2–10% MeOH:CH_2Cl_2 as eluent to give 15 mg (10%) of the ester. The ester (15 mg, 0.02 mmol) was dissolved in MeOH (0.5 mL), aqueous 2N NaOH (1 ml) was added, and the mixture was stirred at room temperature for 5 h. After evaporation of MeOH under reduced pressure, water was added and the mixture was acidified with glacial AcOH to pH 5–6. The suspension was filtered, and the solid was washed with water and dried *in vacuo* to give 12 mg of a yellow solid. Purification by preparative HPLC in a gradient of 30–50% MeCN:H_2O as eluent gave 8 mg (55%) of 17 as the TFA salt. ^{1}H NMR: 10.39 (s, 1H), 8.53 (s, 1H), 8.08 (s, 1H), 7.80 (d, 1H), 7.07 (m, 2H), 6.87 (m, 2H), 4.64 (s, 1H), 4.1 (m, 1H), 3.0–4.0 (m, 12H), 2.53 (q, 2H), 2.35 (m, 2H), 2.17 (s, 3H), 1.17–2.1 (m, 10H), 1.1 (t, 3H). HRMS: calcd for $C_{37}H_{44}FN_6O_6$, 687.3306; found, 687.3316.

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-(3,4-dimethylanilino)uracil, 18

Ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate and 3-(4-iodobutyl)-6-(3,4-dimethylanilino)uracil were reacted as described for 5. Yield: 56%. ^{1}H NMR (DMSO-d_6): 15.22 (s, 1H), 10.37 (s, 1H), 8.66 (s, 1H), 8.06 (s, 1H), 7.92 (d, 1H), 7.55 (d, 1H), 7.13 (d, 1H), 6.83–7.06 (m, 2H), 4.72 (s, 1H), 3.59–3.89 (m, 3H), 2.59 (s, 4H), 2.38 (m, 2H), 2.19 (d, 4H), 0.94–1.64 (m, 8H). HRMS: calcd. for $C_{33}H_{38}FN_6O_5$ 617.2887; found 617.2863,

3-{4-[1-(1-Cyclopropyl-3-carboxy-4-oxo-6-fluoro-7-quinolyl)-4-piperazinyl]butyl}-6-anilinouracil, 19

Ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-piperazinylquinoline-3-carboxylate and 3-(4-iodobutyl)-6-anilinouracil were reacted as described for 5. Yield: 77%. ^{1}H NMR (DMSO-d_6): 15.21 (s, 1H), 10.62 (s, 1H), 8.65 (s, 1H), 8.37 (s, 1H), 7.90 (d, 1H), 7.57 (s, 1H), 7.12–7.50 (m, 5H), 4.82 (s, 1H), 3.70–3.90 (m, 3H), 3.35 (m, 4H), 2.57 (m, 4H), 2.37 (m, 2H), 1.40–1.68 (m, 4H), 1.32 (m, 2H), 1.20 (m, 2H). HRMS: calcd. for $C_{31}H_{34}FN_6O_5$ 589.2574; found 589.2592.

Enzyme Assays

DNA polymerase IIIC (pol IIIC) of *B. subtilis* was the homogenous recombinant protein expressed and prepared as described previously.[10] The enzyme was assayed in a 96-well plate format by using activated calf thymus DNA as a substrate as described.[30] Apparent inhibition constants (K_i values) were determined in a truncated assay lacking the competitor dGTP as described previously.[31] Topoisomerase IV and gyrase from *B. subtilis* were prepared and assayed as described.[11]

Bacterial Strains

The standard panel for determination of Minimum Inhibitory Concentration (MIC) values included *S. aureus* 25923, *S. aureus* 13709 (Smith), *E. faecalis* 29212, and *E. faecium* 19434, all purchased from the American Type Culture Collection (ATCC, Manassas, VA). Methicillin-resistant *S. aureus* (MRSA 1094) and vancomycin-resistant *E. faecium* (VRE 700802) are clinical isolates provided by the University of Massachusetts Medical School. *B. subtilis* (BD54) is a standard laboratory strain. *E. coli* (J53) was provided by Prof. Martin Marinus, University of Massachusetts Medical School.

Minimum Inhibitory Concentration (MIC)

Measurement of Minimum Inhibitory Concentration (MIC) was done as described,[3] by incubating bacteria at 37 °C for 16 to 24 h in the presence of twofold serial dilutions of test compounds. Compounds were dissolved in and diluted from DMSO stocks, and all assays contained 1% DMSO. MIC values are the lowest concentrations of test compounds at which bacterial growth was not apparent (<25% of the DMSO control, based on optical density at 600 nm).

Animal studies

Pathogen-free Swiss-Webster mice (males, 20–24 g) were purchased from Taconic Farms (Germantown, NY). The animals were housed at the University of Massachusetts Medical School (UMMS) Animal Medicine facility. All animal experiments were approved by the UMMS institutional animal care and use committee. Mice were allowed free access to food and water throughout the studies.

Pharmacokinetic studies

Compound 5 was given as an iv bolus dose of 20 mg/kg in DCP to mice by tail vein injection in a volume of 5 mL/Kg. At appropriate times mice were anesthetized with halothane, and blood was collected by cardiac puncture and placed in heparinized tubes. Plasma was harvested, and a 0.3 mL aliquot was mixed with an equal volume of MeCN, and samples were centrifuged to precipitate protein. The supernatant was evaporated (SpeedVac®), and the residue was suspended in 200 μL of 20% MeCN:H_2O for analysis by reverse phase HPLC. Conditions were as previously described.[4] The mobile phase consisted of MeCH:H_2O:NEt_3:AcOH (25:74.7:0.2:0.1) at a flow rate of 1 mL/min and a detection wavelength of 282 nm. The concentration of 5 was determined by comparison of peak areas with those from a standard curve generated from plasma samples spiked with compound. The linear range was 1 to 40 μg/mL. Mass spectra of 5 and its putative metabolite were measured with a ion trap instrument by APC ionization, and samples were introduced by infusion from solutions in water or by HPLC in the above conditions.

Antibacterial efficacy *in vivo*

S. aureus (Smith) was grown at 37 °C to log-phase in Luria Broth (LB), and other organisms were grown in brain-heart infusion broth (BHIB). The colony forming units (CFU) were

determined using a nomogram relating CFU to optical density at 600 nm. Bacteria were washed in fresh cold broth, and given by ip injection to mice as a suspension in 0.5 mL of broth, and with or without 5% mucin as indicated in Tables 3 and 5. Groups of five or ten mice were treated at 15 min post-infection and/or various times post-infection with vehicle, test compound in vehicle, or vancomycin hydrochloride (Vancocin®, Lilly) in saline at 30 mg/Kg. Mice were returned to their cages and monitored for mortality for up to 72 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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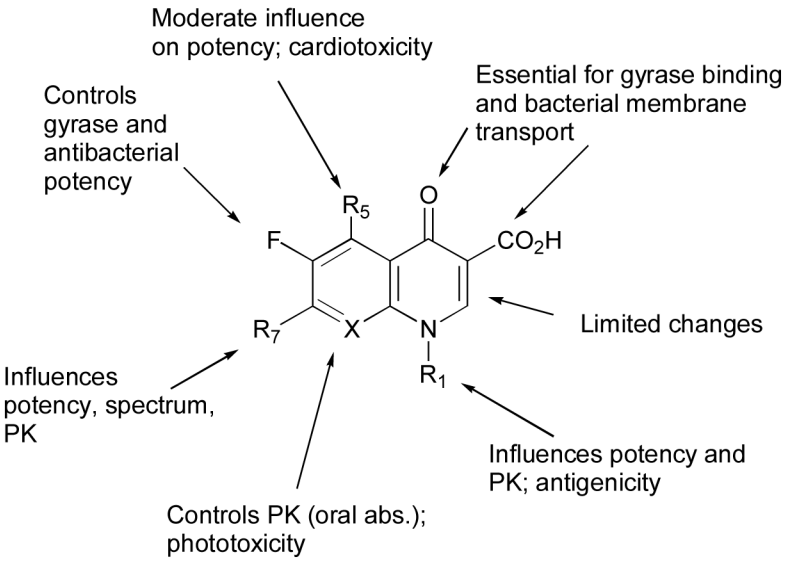


Figure 1.
Overview of SAR of fluoroquinolone antibacterial drugs. PK, pharmacokinetics.

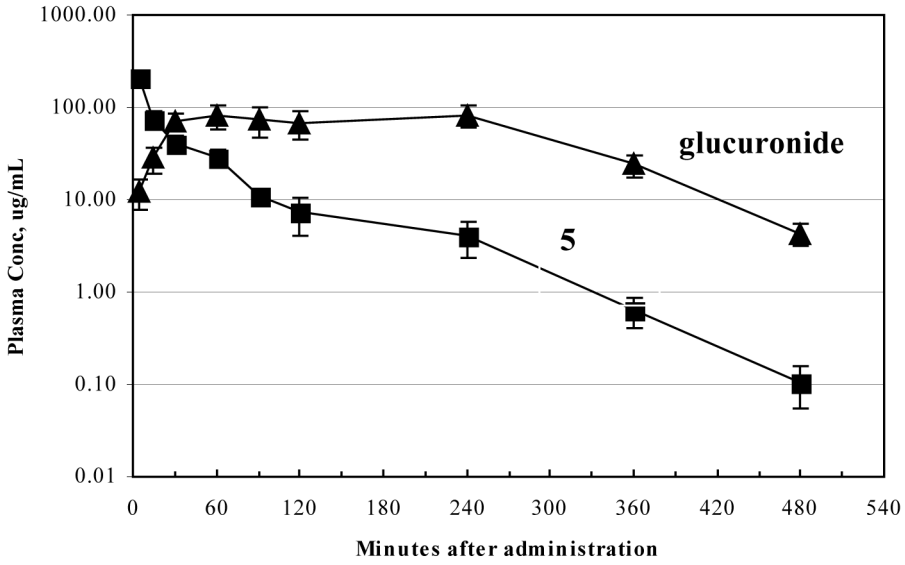
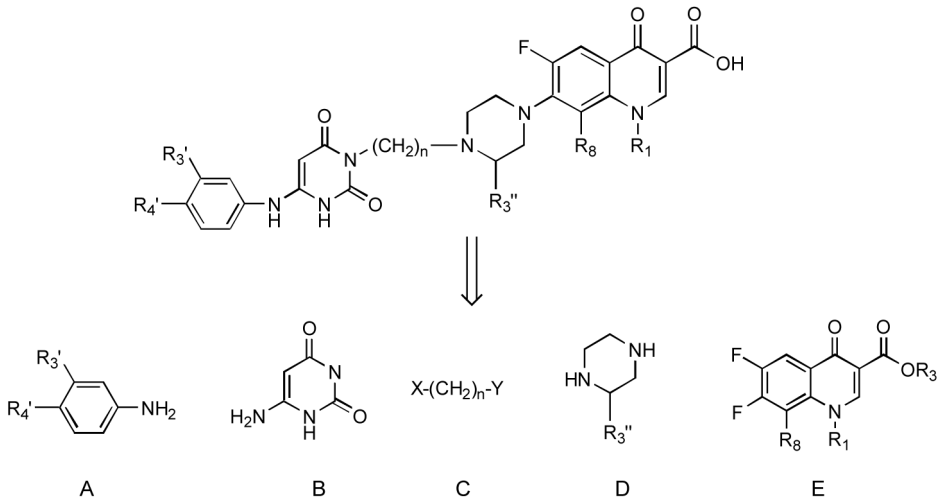
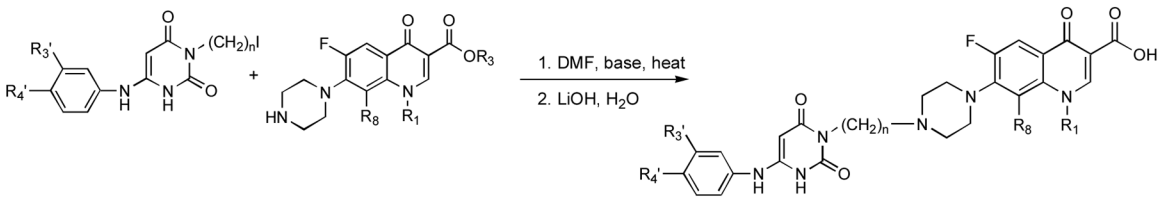


Figure 2.
Mean plasma concentrations of **5** (■) and its glucuronide metabolite (▲) after a 100 mg/kg iv dose of **5** to mice (n = 3).



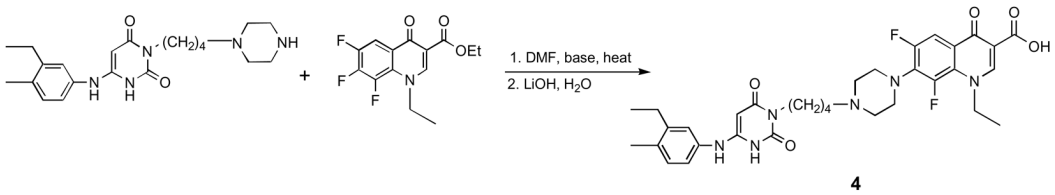
Scheme 1.
Retrosynthetic analysis of AU-FQ hybrids.



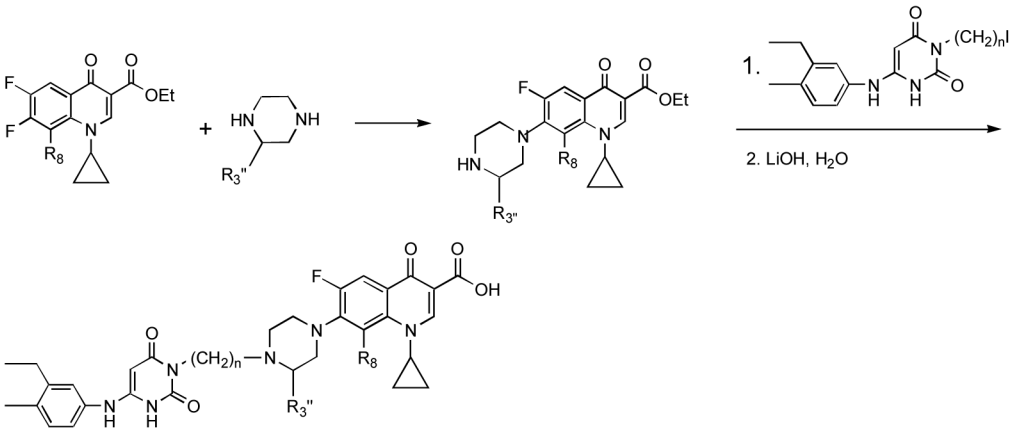
Cpd	R_1	R_8	n	$R_{3'}$	$R_{4'}$
2	Et	H	4	Et	Me
3	Et	(N)	4	Et	Me
5	cPr	H	4	Et	Me
6	cPr	H	5	Et	Me
7	cPr	Cl	4	Et	Me
8*	cPr	OMe	4	Et	Me
9	tBu	H	4	Et	Me
10	2,4-diFPh	H	4	Et	Me
11	4-FPh	H	4	Et	Me
18	cPr	H	4	Me	Me
19	cPr	H	4	H	H

*via BF_2 complex

Scheme 2.

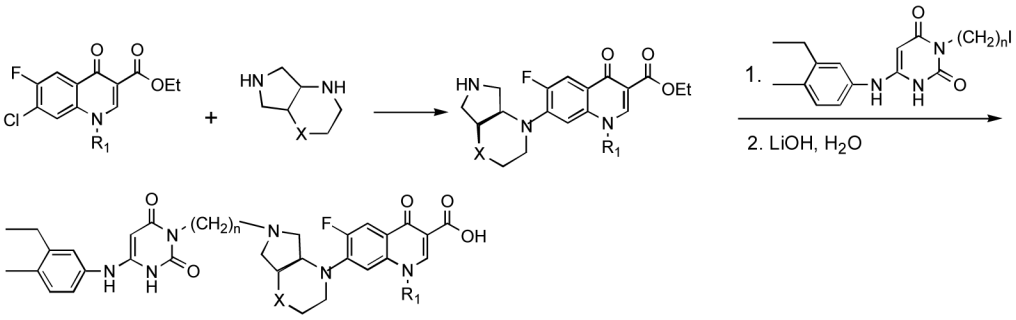


Scheme 3.



Cpd	$R_{3''}$	R_8
12	Me	H
12R	(R)-Me	H
12S	(S)-Me	H
13	Me	F
13R	(R)-Me	F
13S	(S)-Me	F
14	CH_2OH	F

Scheme 4.

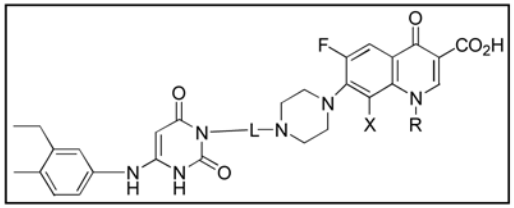
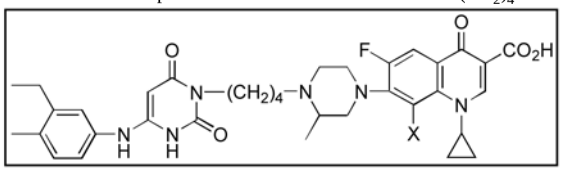
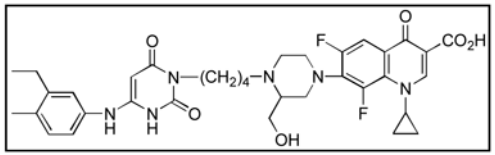


Cpd	R_1	X
15	Et	CH_2
16	cPr	CH_2
17	cPr	O

Scheme 5.

Table 1

Pol IIIC inhibition and antibacterial activity of AU-FQ hybrid compounds

Cpd	R	X	L	K_i(μM)	MIC (μg/ml)							
				B.s. pol IIIC	*B. subtilis* BD54	*S. aureus* 25923	*S.a.*13709 (Smith)	MRSA 1094	*E. faecalis* 29212	*E. faecium* 19434	VRE 700802	*E.coli* J53
1				0.066	1.25	5	5	5	5	5	5	>40
												
2	Et	H	$(CH_2)_4$	0.024	0.313	1.25	1.25	2.5	0.625	0.625	0.625	>40
3	Et	(aza)	$(CH_2)_4$	0.018	0.313	1.25	1.25	2.5	1.25	1.25	0.625	>40
4	Et	F	$(CH_2)_4$	0.021	0.313	1.25	0.625	1.25	1.25	1.25	0.625	>40
5	cPr	H	$(CH_2)_4$	0.024	0.313	1.25	0.625	2.5	0.625	1.25	0.625	>40
6	cPr	H	$(CH_2)_5$	0.019	0.313	1.25	1.25	2.5	0.625	1.25	1.25	>40
7	cPr	Cl	$(CH_2)_4$	0.01	0.078	0.313	0.156	1.25	0.625	1.25	0.625	20
8	cPr	OMe	$(CH_2)_4$	0.014	0.156	0.625	0.313	1.25	1.25	1.25	0.625	>40
9	tBu	H	$(CH_2)_4$.HCl	0.013	0.313	0.625	0.625	1.25	0.625	1.25	1.25	>40
10	diFPh	H	$(CH_2)_4$	0.018	0.313	0.625	0.313	1.25	1.25	1.25	1.25	>40
11	pFPh	H	$(CH_2)_4$	0.017	0.156	1.25	0.625	2.5	0.625	1.25	1.25	>40
												
12		H		0.026	0.313	0.625	0.625	1.25	1.25	1.25	0.626	40
12R		H		0.012	0.313	1.25	0.625	2.5	0.625	0.625	0.625	10
12S		H		0.011	0.156	1.25	0.625	1.25	0.625	1.25	0.625	40
13		F		0.019	0.156	0.313	0.156	1.25	1.25	1.25	0.625	5
13R		F		0.007	0.156	0.313	0.156	1.25	1.25	2.5	1.25	5
13S		F		0.004	0.156	0.625	0.625	0.625	1.25	1.25	0.625	20
												
14				0.011	0.625	1.25	0.625	5	2.5	2.5	2.5	>40

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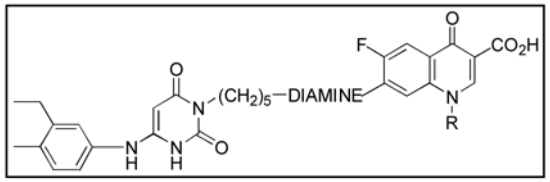
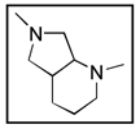
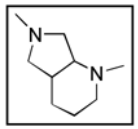
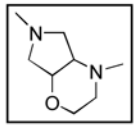
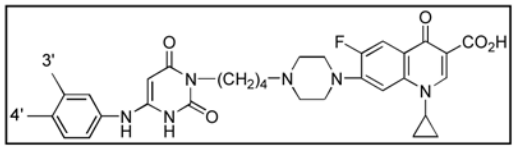
Cpd	R	X	L	K_i(μM) *B.s.* pol IIIC	MIC (μg/ml) *B. subtilis* BD54	*S. aureus* 25923	*S.a.*13709 (Smith)	MRSA 1094	*E. faecalis* 29212	*E. faecium* 19434	VRE 700802	*E.coli* J53
												
15	Et			0.037	2.5	10	5	>80	10	20	5	>80
16	cPr			0.041	1.25	2.5	1.25	>80	>80	>80	>80	>80
17	cPr			0.033	0.625	1.25	0.625	5	5	>80	10	40
												
	3′	4′										
18	Me	Me		0.095	0.313	1.25	0.625	5	2.5	2.5	1.25	>40
19	H	H		29	2.5	2.5	2.5	>40	10	>40	40	>40
norfloxacin				inact	0.313	0.625	0.156	40	2.5	10	80	1.25
ciprofloxacin				inact	0.156	0.313	0.156	20	0.625	10	80	0.313
sparfloxacin				inact	<0.078	<0.078	<0.078	10	5	1.25	80	<0.078
linezolid				inact	0.625	1.25	2.5	1.25	1.25	2.5	1.25	>80

Table 2

Inhibition of *B. subtilis* topoisomerases by selected compounds.

	IC_{50}(μg/ml)*	
Cpd	**gyrase**	**topo**
5	28.4	22.9
13	21.7	28.9
13S	9.2	13.2
13R	11.7	24.5
nalidixic acid	34.6	276
ciprofloxacin	1.9	0.74
sparfloxacin	0.4	0.5

* see Experimental Section for assay details.

Table 3

Effect of **5** given iv on survival of *S. aureus* (Smith) ip infected mice.

Treatment	Dose (mg/kg)	Survivors (n = 10)[a]
Vehicle[b]	− (5 mL/Kg)	0
Vancomycin[c]	30	10
5	50	7
“	75	10
“	100	10

[a] Groups of 10 mice were infected ip with 10^8 CFU *S. aureus* (Smith) and dosed iv 15 minutes later; survivors were counted at 72 hours post infection.

[b] DCP.

[c] in saline.

Table 4

Effect of single iv doses of AU-FQ hybrids on survival of mice after ip infection with *S. aureus* (Smith).

Cpd*	ED_{50} (mg/Kg)
8	25
10	27
12	20
12R	20
12S	30
13	15
13R	20
13S	15
14	35

* via tail vein in DCP vehicle, dosed at 5 mL/Kg, 15 min post-infection

Table 5

Efficacy of **13** against Gram+ infections in mice.

Organism	Inoculum, CFU/mouse[a]	Dose, mg/kg[b]	Doses, (min post-infection)	Survivors/treated at 72 hours	% survival at 72 hours
MRSA 1094	1×10^7	-	-	0/20	0
		25	2 (15,135)	1/20	5
		50	2 (15, 135)	0/5	0
		75	2 (15, 135)	11/20	55*
		Vanco, 30	1 (15)	20/20	100**
***E. faecalis* 29212**	3×10^7	-	-	0/15	0
		25	1 (15)	2/15	13
		50	1 (15)	2/5	40
		75	1 (15)	4/5	80*
		25	2 (15, 135)	2/5	40
		50	2 (15, 135)	8/10	80**
		Vanco, 100	1 (15)	15/15	100**
VRE 700802	3×10^8	-	-	0/20	0
		100	1 (15)	2/20	10
		50	2 (15, 135)	1/10	10
		75	2 (15, 135)	0/5	0
		50	2 (15, 195)	0/5	0
		75	2 (15, 195)	9/20	45*
		Cipro, 100	1 (15)	14/15	93**

[a] ip in 0.5 mL BHIB + 5% mucin.

[b] iv via tail vein in DCP; vancomycin and ciprofloxacin in saline; dose volume 5 mL/Kg.

* $p<0.05$

** $p<0.001$