Discovery of Novel Quinoline-Based Estrogen Receptor Ligands Using Peptide Interaction Profiling

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Traditional approaches to discovery of selective estrogen receptor modulators (SERMs) have relied on ER binding and cell-based estrogen response element-driven assays to identify compounds that are osteoprotective but nonproliferative in breast and uterine tissues. To discover new classes of potential SERMs, we have employed a cell-free microsphere-based binding assay to rapidly characterize ERα interactions with conformation-sensing cofactor or phage display peptides. Peptide profiles of constrained triarenes were compared to known proliferative and nonproliferative ER ligands to discover potent quinoline-based ligands with minimal Ishikawa cell stimulation.

Introduction

The biological effects of estrogen are mediated by two nuclear hormone receptors, ERα and ERβ.[1−4] Several studies suggest that ERα is primarily responsible for much of the pharmacological effects of estrogen and estrogen hormone replacement therapy.[5] The role of ERβ is not clearly understood, although it may contribute to estrogen pharmacology in the central nervous system and in inflammatory responses.[6,7] The binding of endogenous estrogens to their receptors results in conformational changes in the ligand binding domain (LBD) that facilitate the recruitment of coactivator proteins and aid the assembly of a transcription activation complex.[3] Synthetic ER ligands can exhibit a range of functional activity, from agonist to antagonist, that is tissue and cell-type dependent.[4] These nonsteroidal ligands, which are known as selective estrogen receptor modulators (SERMs), can mimic the therapeutic effects of hormone replacement therapy in prevention of osteoporosis while opposing the action of estrogens in reproductive tissues.

Triaryl template-based SERMs emerged in the 1960s with the discovery of the breast cancer therapeutic tamoxifen.[1,2] Unfortunately, tamoxifen demonstrated too much residual uterine stimulation for use in postmenopausal women for treatment of osteoporosis. A conformationally constrained benzothiophene analogue, raloxifene, was developed as a breast cancer therapeutic with less uterotrophic activity and was subsequently approved as a second generation SERM for prevention of osteoporosis in postmenopausal women.[2]

While the hydroxyl groups in 4-hydroxy-tamoxifen (4-OHT) and raloxifene serve as the primary pharmacophores for recognition by the LBD, the receptor modulatory effects are attributed to interaction of the ethanolamine side chains with the C-terminal AF-2 helix and Asp-351 on helix-3 of ERα.[1,8,9] We previously described identification of the second generation SERM GW5638 and its 4-hydroxylated metabolite GW7604.[10] These SERMs were unique in their replacement of the ethanolamine side chains with an acrylic acid moiety and were particularly effective in models of tamoxifen-resistant breast cancer. As part of a program to discover third generation SERMs that show increased efficacy in postmenopausal diseases while maintaining low uterotrophic activity, we chose to synthesize constrained triarenes containing the acrylic acid side chain. To this end, the parallel synthesis and biological evaluation of new quinoline-based ER ligands (**1**) are disclosed herein.

Standard approaches toward the discovery of SERMs have relied on ER binding and estrogen response element (ERE)-based functional readouts. Agonist activity in cell-based ERE assays has been correlated with cell proliferation in breast and uterus but is unable to predict the complex pharmacology of SERMs.[11] Recent studies have demonstrated that recombinant phage peptide libraries provide an alternative approach to monitor ligand induced conformational changes in ERα.[12] For our ER program, we opted to use a microsphere-based in vitro binding assay to characterize multiplexed interactions of ERα with coactivator and corepressor-based or phage display-derived peptides in the presence of both standard and novel quinoline ligands. The multiplexed microsphere assay allows simultaneous monitoring of up to 100 peptide interactions with an ERα/ligand complex.[13,14]

Peptide profiles of known ER standards and SERMs (17β-estradiol, 4-OHT, raloxifene, idoxifene, and GW7604) were determined and correlations established with standard cell-based phenotypic assays.[14] Quinoline-based ERα ligands[15] were first evaluated in a competition binding assay and subsequently profiled against a set of ≥50 cofactor and phage display-derived peptides. Confirmatory follow up in an Ishikawa uterine cell stimulation assay was included in the analysis. The results of these studies are discussed below.

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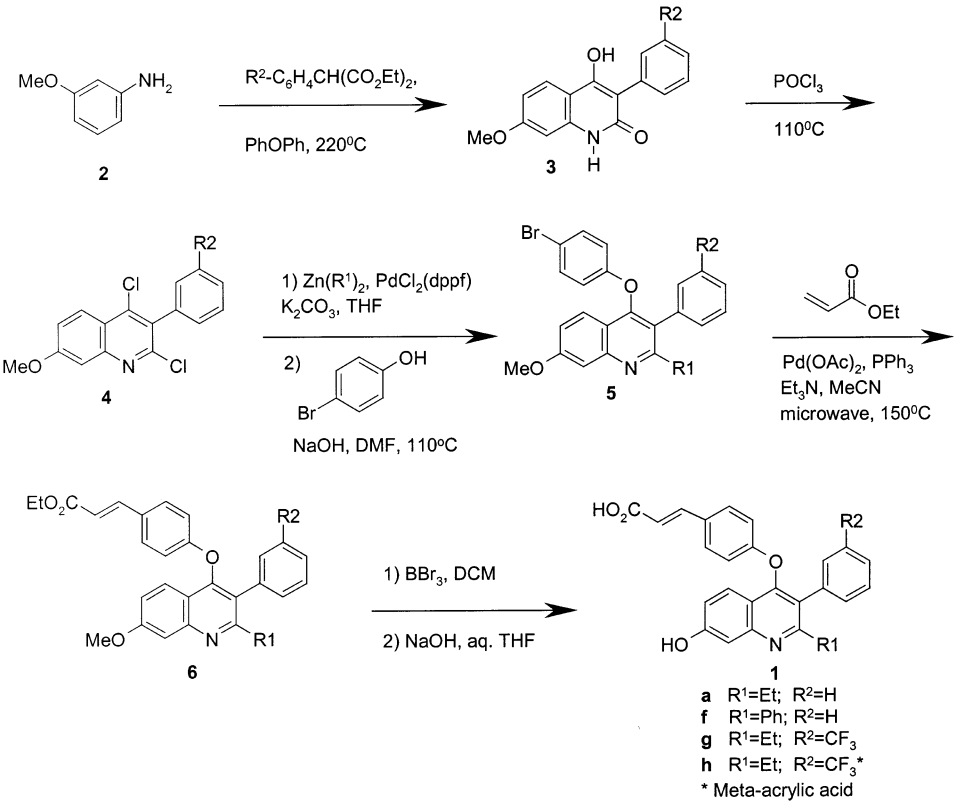
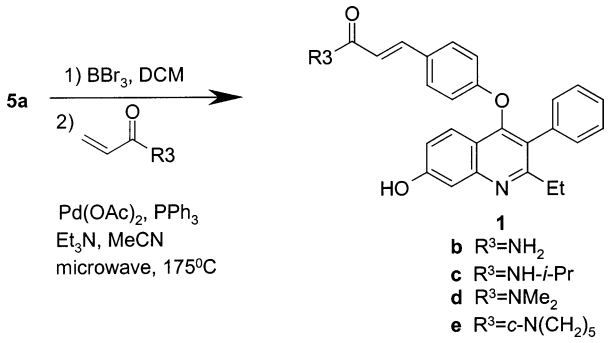
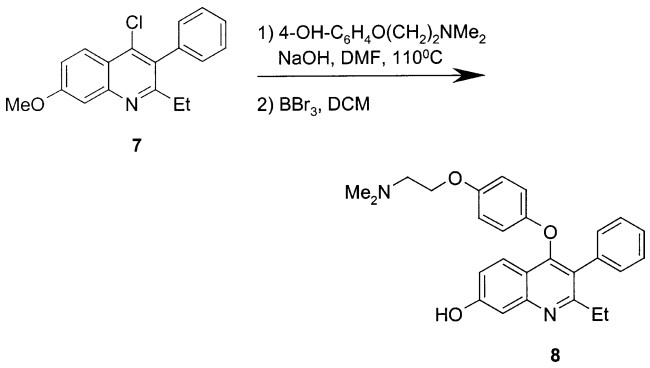
Scheme 1. Parallel Synthesis of Quinolinyl-acrylic Acid-Based ER Ligands **1a**,**f**−**h**



Scheme 2. Synthesis of the Quinolinyl-acrylamide-Based ER Ligands **1b**−**e**



Scheme 3. Synthesis of the Quinolinyl-ethanolamine **8**



Chemistry. Quinoline targets were prepared starting from aniline **2** (Scheme 1). Aniline **2** was condensed with aryl-substituted diethyl malonates at elevated temperature to afford quinolones **3**.[16] Conversion of **3** to dichloroquinolines **4** in phosphorus oxychloride followed by coupling with $(R^1)_2Zn$ and displacement with 4-bromophenol afforded intermediates **5** (compound **1h** was prepared using 3-bromophenol). Heck coupling [palladium(II) acetate, triethylamine, triphenylphosphine, acetonitrile, microwave irradiation at 150 °C] of **5** with ethyl acrylate furnished **6**. The methyl ether may be unmasked with boron tribromide to provide phenol ester intermediates.[17] For acrylic acid products, the corresponding esters were saponified with sodium hydroxide to give products **1a**,**f**−**h**.

Acrylamide products related to carboxylic acid **1a** were prepared as follows (Scheme 2). Aryl bromide **5a** was demethylated using the standard boron tribromide conditions to give the corresponding hydroxyquinolines, and the hydroxyquinolines were coupled with variably substituted acrylamide reagents using Heck methodology to furnish acrylamide products **1b**−**e**. Quinoline-based ethanolamine analogue **8** was prepared in three steps starting from 4-methoxyphenol and *N*,*N*-dimethyl-2-chloroethylamine (Scheme 3).[18] *N*,*N*-Dimethyl 4-hydroxyphenyl-2-oxyethylamine was alkylated with 4-chloroquinoline **7**, and the resultant methoxyquinoline product was demethylated with boron tribromide to furnish **8**.

Results and Discussion

Quinoline analogues **1a**−**h** and **8** were tested in ERα and ERβ scintillation proximity assay (SPA) binding (Table 1).[14] Compounds **1a**−**h** bound ERα with pK_is ranging from 5.8 (*m*-cinnamic acid **1h**) to 8.0 (*N*-isopropyl-*p*-cinnamide **1c**). Ethanolamine analogue **8** showed high affinity (pK_i = 8.1) for ERα. The quinoline series members did not exhibit significant subtype selectivity for ERα or ERβ.

Quinolines with binding affinities of pK_i > 6 were profiled against a standard set of ca. 50 cofactor and

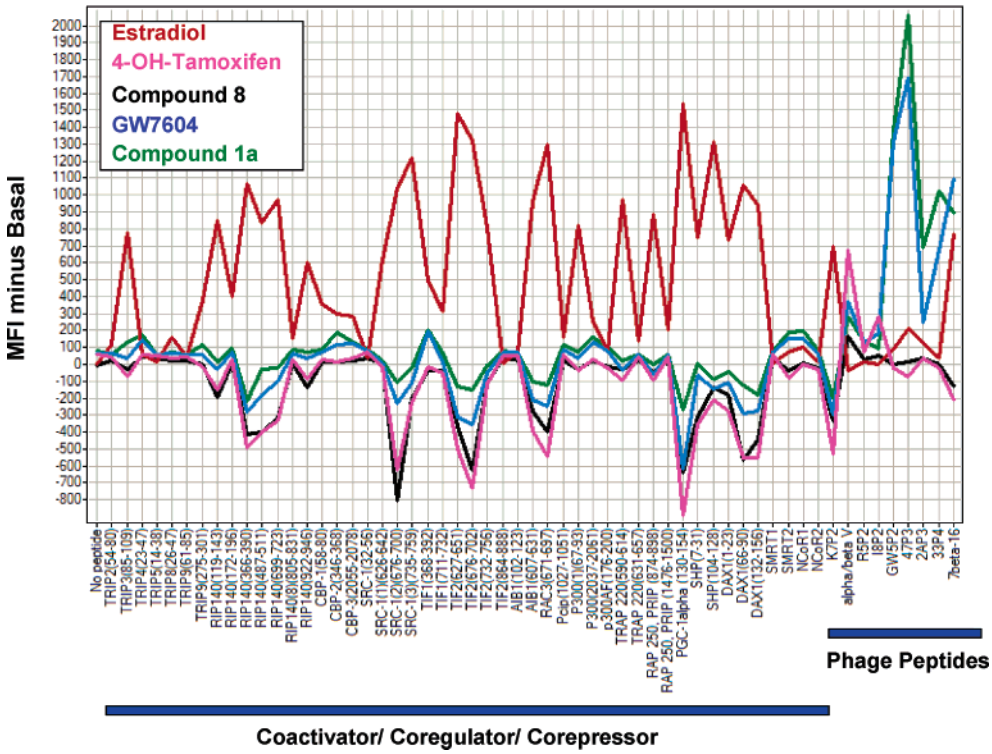
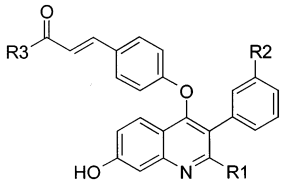


Figure 1. Comparison of compound effects on ERα binding to cofactor and phage display peptides. Binding of compound bound ERα to the respective fluorescent microsphere-affixed peptides was measured using flow cytometry. Profile plots show the maximum/minimum values via a traditional histogram. The *y*-axis depicts the mean fluorescence intensity (MFI) for Alexa-labeled ERα-binding (10 nM) in the presence of compound (10 μM) minus the basal fluorescence.

Table 1. ERα/ERβ Binding, GW5P2 Peptide Binding, and Ishikawa Cell Stimulation Data for Quinolines



1a-h

				binding pK_i[a]		peptide MFI[b]	Ishikawa cell	
compd	R1	R2	R3	ERα	ERβ	GW5P2	pIC$_{50}$	% E2[c]
1a	Et	H	OH	6.9	7.2	1380	6.9	<1
1b	Et	H	NH_2	7.8	7.6	540	7.1	8
1c	Et	H	NH-*i*Pr	8.0	7.8	710	7.7	1
1d	Et	H	$N(CH_3)_2$	8.0	7.7	350	8.4	12
1e	Et	H	*c*-$N(CH_2)_5$	7.6	7.6	390	8.4	6
1f	Ph	H	OH	5.5	6.2	NT[e]	5.6	9
1g	Et	CF3	OH	7.0	6.6	1590	8.1	<1
1h[d]	Et	H	OH	5.8	5.8	NT	NT	NT
8[f]				8.1	8.0	0	8.5	29
17β-estradiol				8.7	8.5	190	NT	100
4-OH-tamoxifen				8.6	8.5	−20	8.8	14
GW7604				8.6	8.0	1320	8.9	4

[a] SPA binding (n ≥ 2; ±20%). [b] Mean fluorescence intensity (MFI) corrected to basal for binding of ERα/ligand to the GW5638 selective peptide (standards, $n = 20$; quinolines, $n = 1$). [c] Ishikawa cell alkaline phosphatase activity was measured as percent maximal induction compared to 17β-estradiol (E2; 100%) ($n = 2$); pIC$_{50}$s were determined against 1 nM estradiol-induced stimulation. [d] *m*-CH=$CHCO_2H$. [e] NT = not tested. [f] See Scheme 3.

phage display-derived peptides in the microsphere-based in vitro binding assay (Figure 1). Prior studies had identified phage-generated peptides selective for 17β-estradiol/agonists (K7P2), 4-OHT (alpha/betaV),[19] GW7604 (GW5P2), idoxifene (I8P2), and raloxifene (R5P2). Two additional GW7604-selective peptides, 47P3 and 33P4, were identified using a second generation phage display library.[13,14] 17β-Estradiol recruited coactivator NR box peptides derived from RIP140(699−723), RIP140(922−946), TIF2(676−702), PGC-1α(130−154), and SHP(7−31) (Figure). The recruitment of the alpha/betaV peptide to ERα induced by 4-OHT distinguished it from raloxifene and provided a potential surrogate marker for uterotrophic activity. Potential third generation SERMs were defined as those compounds that showed a distinct peptide profile compared to 17β-estradiol and 4-OHT.

Quinoline compounds **1** exhibited peptide profiles distinct from 17β-estradiol and 4-OHT. Acrylic acids **1a** and **1g** recruited the GW5P2 peptide with affinity similar to GW7604, while binding of **1a** to K7P2 and alpha/betaV was minimal (Table 1, Figure 1). Due to their low ERα affinity, the peptide profiles of **1f** and **1h** were not determined. Amides **1b**−**e** exhibited diminished recruitment of the GW5P2 peptide compared to the corresponding acid **1a**. Ethanolamine analogue **8** produced only basal interaction with GW5P2 peptide and, interestingly, recruited alpha/betaV peptide minimally (Figure 1). However, the overall peptide profile of **8** was more similar to 4-OHT than acid **1a**.

To confirm the predictions from the peptide profiling assay, the quinoline compounds were tested in Ishikawa cells for induction of endogenous alkaline phosphatase activity (Table 1).[14] Notably, the compounds with the strongest recruitment of GW5P2 peptide, acrylic acids **1a** and **1g**, demonstrated the lowest induction of uterine

cell activity. The quinoline **8** containing the ethanolamine side chain stimulated Ishikawa cell activity, consistent with its 4-OHT-like peptide profile. Previous in vivo evaluation of the acrylic acid bearing triphenylethylene GW5638 in OVX rats suggests that quinoline congeners such as **1**, exhibiting a similar peptide profile to GW5638, might be correspondingly bone-protective. GW5638, dosed at 1 mg/kg, demonstrated bone loss prevention equivalent to estradiol.[10] A pharmacokinetic study of a quinoline representative was performed to evaluate in vivo exposure. Quinoline **1a** exhibited bioavailability of 11%, plasma half-life of 3.8 h, and total body clearance of 84 mL/min/kg upon oral administration to rats (3 mg/kg, po).[20] Pharmacological evaluation of quinoline-based ER ligands in OVX rats will be reported in due course.

In summary, we have employed a peptide profiling assay to identify potential third generation SERMs from a quinoline template that demonstrate low stimulation of uterine cell proliferation. Interestingly, the acrylic acid pharmacophore's ERα conformation correlates with this phenotype independent of chemical template (see GW7604 and quinoline **1a**). Thus, peptide profiling of additional chemotypes offers the potential to identify and classify new SERMs.

Experimental Section

In Vitro Procedures. Scintillation proximity assays to measure ERα and ERβ binding were performed as published ($n > 2$).[14] The stimulation of human Ishikawa endometrial cells was determined by increase in intrinsic alkaline phosphatase activity.[14] Compounds were tested for potential to stimulate the cells compared to 17β-estradiol. The percent E_{max} was defined as 100x (spectrophotometric reading at 405 nm with compound-blank)/(spectrophotometric reading at 405 nm with 17β-estradiol-blank). Compounds were also tested for inhibition of 17β-estradiol (1 nM) stimulated alkaline phosphatase activity and recorded as pIC_{50}s ($n = 2$).

Peptide Profiling Studies. All components (microsphere-attached peptides, 10 μM ligand, Alexa 532-labeled ERα LBD amino acids 299−554) were combined in wells of a 96-well microtiter plate in total volume of 75 μL.[13,14] Each well contained 3000 microspheres of a given population and ≤60 individual microsphere populations. Suspensions were incubated for 1.5−2.0 h at room temperature in the dark. Fluorescence for each microsphere was measured by flow cytometry ($n = 2$). For experimental details, see ref 14.

Chemical Procedures. Analytical data for compounds **1c**−**h** appear in the Supporting Information. ^{1}H NMR spectra were recorded on a Varian VXR-300, a Varian Unity-300, a Varian Unity-400 instrument, or a General Electric QE-300. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants are hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Compounds were analyzed on a Micromass Quattro Micro QAA075 LC/MS (electrospray ionization) using either Conditions I or Conditions II (below). Retention times were recorded for each compound.

Conditions I. The column employed was a 50 × 2.0 mm Synergi Max-RP (4 microns). Mobile phase was 85/15 water/MeOH to 100% MeOH with a 4 min run time, holding at 100% MeOH for final 2 min. Water contained 0.1% v/v formic acid, MeOH contained 0.075% v/v formic acid. The flow rate was 0.8 mL/min with 3 μL of solution injected.

Conditions II. Conditions were identical to Conditions I except the mobile phase employed was 98/2 water/MeOH to 100% MeOH.

Final compounds were purified on an Agilent-1100 HPLC with a 5 μm Phenomenex Luna C-18(2), 150 × 21.2 mm column; with gradient of 10−90% MeCN/water/0.1% TFA was run over 10 min, followed by a 4 min organic wash. Flow rate was 20 mL/min with DAD/254 nm.

Materials. Reagents were purchased from Acros, Aldrich, or Fluka Chemical Co.

3-Phenyl-4-hydroxy-7-methoxyquinol-2-one (3a). A dark solution of 3-anisidine (5.37 g, 44.8 mmol), diethyl 2-phenylmalonate (10.57 g, 44.8 mmol), and Ph_2O (50 mL) was heated at 220 °C for 1 h without a reflux condenser to allow EtOH evaporation. The reaction was cooled to room temperature, and the resultant gray ppt filtered and washed with Et_2O and dried to afford **3a** (11.0 g, R2 = H in Scheme 1, 92%): MS $(M + H)^+$ 269.28.

2,4-Dichoro-3-phenyl-7-methoxyquinoline (4a). A slurry of **3a** (7.0 g, 26.2 mmol) and $POCl_3$ (30 mL) was heated at 110° C for 2 h, cooled to room temperature, and stored at 0 °C for 1 h. This mixture was decanted into ice cold 10% K_2CO_3 (50 mL) to furnish a white ppt. The ppt was filtered and partitioned between 10% K_2CO_3 (100 mL) and EtOAc (150 mL). This mixture was stirred for 1 h, filtered, and the white powder dried to afford **4a** (7.3 g, 91% yield): MS $(M + H)^+$ 304.02.

2-Ethyl-3-phenyl-4-(4-bromophenyloxy)-7-methoxyquinoline (5a). To a solution of **4a** (2.99 g, 9.83 mmol), K_2CO_3 (4.076 g, 29.49 mmol), and dichloro[1,1′-bis (diphenylphosphino)ferrocene]palladium(II) dichloromethane adduct (0.80 g, 0.98 mmol) in THF (60 mL) was added Et_2Zn (10 mL, 1.0 M in hexanes, 10.0 mmol). The reaction was heated to reflux for 6 h, then cooled to ambient temperature, poured into a saturated NH_4Cl solution (50 mL), and extracted with EtOAc (3 × 40 mL). The combined organic layer was washed with brine (50 mL), dried over $MgSO_4$, and evaporated. The crude solid was purified over silica gel-60 via MPLC (EtOAc/hexane) to afford 2.64 g (91% yield) of a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.10−8.07 (s, 1H), 7.57−7.45 (m, 4H), 7.38−7.34 (m, 3H), 3.96 (s, 3H), 2.67−2.60 (q, 2H), 1.16−1.11 (t, 3H). MS $(M + H)^+$ 298.07. A Pyrex screw cap tube (25 × 200 mm) was charged with yellow solid (1.18 g, 3.97 mmol), NaOH (0.794 g, 19.85 mmol), and 4-bromophenol (1.37 g, 7.94 mmol) in DMF (10 mL). The reaction was heated to 110°C for 24 h and cooled to ambient temperature. The reaction was poured into water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine (40 mL), dried over $MgSO_4$, and evaporated. The crude solid was purified over silica gel-60 via MPLC (EtOAc/hexane) to afford **5a** (0.898 g, 52%) as an off-white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.76−7.73 (d, 1H), 7.49 (s, 1H), 7.33−7.28 (m, 3H), 7.22−7.19 (d, 2H), 7.16−7.10 (m, 3H), 6.50−6.47 (d, 2H), 3.99 (s, 3H), 2.85−2.78 (q, 2H), 1.25−1.20 (t, 3H). MS $(M + H)^+$ 434.07.

Ethyl 3-(4-[(2-Ethyl-7-methoxy-3-phenyl-4-quinolinyl)-oxyphenyl])-2-propenoate (6a). A CEM microwave tube was charged with **5a** (0. 750 g, 1.73 mmol), PPh_3 (0.243 g, 0.80 mmol), palladium acetate (0.097 g, 0.43 mmol), ethyl acrylate (0.56 mL, 5.18 mmol), and Et_3N (0.73 mL, 5.18 mmol) in MeCN (5 mL). The reaction was irradiated (μwave 150 W, 30 min, 150°C), evaporated, treated with water (10 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with brine (20 mL), dried over $MgSO_4$, and evaporated. The crude solid was purified over silica gel-60 via MPLC (EtOAc/hexane) to afford **6a** (0.59 g, 76% yield) as a yellow solid. ^{1}H NMR (300 MHz, C*D*Cl_3): δ 7.74−7.71 (d, 1H), 7.58−7.53 (d, 1H), 7.49−7.48 (d, 1H), 7.28−7.25 (m, 3H), 7.16−7.07 (m, 3H), 6.61−6.58 (d, 2H), 6.28−6.22 (d, 1H), 4.26−4.19 (q, 4H), 3.95 (s, 3H), 2.85−2.77 (q, 2H), 1.33−1.28 (t, 3H), 1.24−1.19 (t, 3H). MS $(M + H)^+$ 454.19.

3-(4-[(2-Ethyl-7-hydroxy-3-phenyl-4-quinolinyl)oxyphenyl])-2-propenoic Acid (1a). A solution of **6a** (0.300 g, 0.65 mmol) in DCM (10 mL) was cooled to −20°C in a MeOH/ice bath. Under nitrogen, BBr_3 was added slowly (6 mL, 1.0 M in DCM, 6.0 mmol). After 3 h the reaction was warmed to ambient temperature, quenched with MeOH (20 mL), and evaporated to afford a crude solid. A solution of this solid in THF (5 mL) was transferred to a Pyrex tube (16 × 125 mm), and NaOH (10.0 mL, 1.0*N*) was added. The reaction was heated to 50°C for 18 h, evaporated, poured into water (10 mL),

adjusted to pH 2 with HCl (1.0 N), and extracted with EtOAc (3 × 5 mL). The combined organic layer was washed with brine (20 mL), dried over $MgSO_4$, and evaporated. The crude solid was purified over silica gel-60 via MPLC (EtOAc/hexane) to afford **1a** (0.043 g,16% yield over two steps; 96% purity) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.59−7.49 (m, 3H), 7.44 (s, H), 7.36−7.25 (m, 6H), 7.09−7.06 (m, 1H), 6.72−6.69 (d, 2H), 6.36−6.31 (d, 1H), 2.71−2.63 (q, 2H), 1.17−1.12 (t, 3H). LC/MS t_R 4.20 min (Conditions I) MS $(M + H)^+$ 412.15.

3-(4-[(7-Hydroxy-2-ethyl-3-phenyl-4-quinolinyl)oxyphenyl])-2-propenoamide (1b). A CEM microwave tube was charged with the phenol analogue of **5a** (0.040 g, 0.095 mmol; prepared by BBr_3 treatment of **5a**, using conditions disclosed for **1a** above), PPh_3 (0.0137 g, 0.045 mmol), palladium(II) acetate (0.005 g, 0.024 mmol), acrylamide (0.032 g, 0.46 mmol), and Et_3N (0.0387 mL, 0.29 mmol) in MeCN (2 mL). The reaction was irradiated (150 W, 30 min, 175°C), filtered, evaporated, poured into water (3 mL), and extracted with EtOAc (3 × 5 mL). The combined organic layer was washed with brine (5 mL), dried over $MgSO_4$, and evaporated. The crude solid was purified via HPLC (MeCN/H_2O) to afford **1b** (0.021 g, 54% yield; 97% purity) as a yellow solid. ^{1}H NMR (300 MHz, C*D*$_3$O*D*): δ 7.73 (d, 1H), 7.35 (m, 7H), 7.20 (m, 2H), 7.10 (dd, 2H), 6.63 (d, 2H), 6.46 (d, 1H), 2.78 (q, 2H), 1.15 (t, 3H). LC/MS t_R 3.05 min (Conditions II); MS $(M + H)^+$ 411.16.

***N,N*-Dimethyl 4-hydroxyphenyl-2-oxyethylamine (CAS no. 100238-29-7).** *N,N*-Dimethyl 4-hydroxyphenyl-oxyethylamine[18] (7.26 mmol) was prepared in two steps from *N,N*-dimethyl-2-chloroethylamine HCl (14.6 mmol) and 4-methoxyphenol (13.3 mmol; Cs_2CO_3, DMF, 80 °C, 4 h conditions) followed by BBr_3-mediated demethylation (DCM, 0 °C, 4 h): ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.48, (bs, 1H), 6.81 (d, 2H), 6.68 (d, 2H), 4.17 (t, 2H), 3.44 (q, 2H), 2.83 (d, 6H). MS $(M + H)^+$ 182.00. $C_{10}H_{15}NO_2$.

***N,N*-Dimethyl 4-[(2-Ethyl-7-hydroxy-3-phenyl-4-quinolinyl)oxyphenyl]-2-oxyethylamine (8).** Compound **8** (0.0148 g, 16% yield from 4-methoxyphenol; 99% purity) was prepared as described previously by reaction of **7** (0.86 mmol) and *N,N*-dimethyl-4-hydroxyphenyl-oxyethylamine (1.73 mmol; see conditions for **5a**) followed by BBr_3-mediated demethylation ((DCM, −20 °C to room temperature, 4 h, see **1a**): mp 184−186°C. ^{1}H NMR (300 MHz, *CDCl*$_3$): δ 8.15 (d, 1H), 7.48 (d, 1H), 7.37 (dd, 1H), 7.30 (m, 3H), 7.18 (m, 2H), 6.78 (m, 2H), 6.67 (m, 2H), 4.23 (t, 2H), 3.54 (t, 2H), 2.96 (s, 6H), 2.90 (q, 2H), 1.19 (t, 3H). LC/MS t_R 1.40 min (Conditions I); MS $(M + H)^+$ 429.04. $C_{27}H_{28}N_2O_3$.

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Supporting Information Available: Analyical data for **1c**−**h**. This material is available free of charge via the Internet at http://pubs.acs.org.

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