Light-Activated Transcription and Repression by Using Photocaged SERMs

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Recently developed methods to regulate the spatial and temporal patterning of genes in a light-directed manner hold promise as powerful tools for exploring the function of genes that act through their unique spatiotemporal patterning. To further explore the application of photocaged ligands of nuclear receptors to control gene expression patterning, the actions of photocaged analogues of selective estrogen-receptor modulators (SERMs) have been evaluated. Photocaged derivatives of hydroxytamoxifen (NB-Htam) and guanidine tamoxifen (NB-Gtam) have been synthesized that selectively antagonize ERα- and ERβ-mediated transcription at classic estrogen response elements (EREs) in response to light. When present only intracellularly, Htam and Gtam provide a similar transient repression response. When SERMs are allowed to diffuse out of the cell, transcription is recovered at a similar rate for Htam and Gtam (6.4 and 5.6 h^{-1}), but is notably faster than is observed with the covalently binding SERM tamoxifen aziridine (Taz) (3.8 h^{-1}). This suggests that the duration of agonist action is controlled by ligand off-rates/diffusion and not by receptor turnover. Gtam activates ERβ-mediated transcription at AP1 sites in a similar way to what has previously been reported for Htam. NB-Gtam and NB-Tam provide a light-activated transcription response at AP1-driven reporters, thus illustrating the unique ability of photocaged SERMs to simultaneously mediate light-activated transcription and repression.

Introduction

The actions of many gene products are elicited through their unique spatial and temporal patterns of expression during development and homeostasis. It is often difficult to study the functions of such gene products as a result of the general lack of methods available to control gene expression in defined spatial and temporal patterns. Several recent reports have described new approaches to spatially and temporally control gene expression with the action of light. These include the use of photocaged forms of enzymes, substrates, or inhibitors involved in signal transduction pathways, photocaged forms of nucleic acids, and a recently reported plant-phytochrome-based yeast two-hybrid.[1–13] New methods for regulating gene expression in spatial and temporal patterns may provide powerful new tools for understanding critical biological phenomenon. Recently, we initiated a program to explore the use of photocaged ligands of the nuclear and steroid hormone receptors as a general method to provide spatial and temporal control of gene expression.[14,15]

Nuclear and steroid hormone receptors function as ligand-dependent transcriptional regulators to control the expression of a diverse array of genes involved in development and homeostasis. We have shown that photocaged analogues of estrogen receptor, retinoic acid receptor, and the thyroid hormone receptor agonists can mediate exposure-dependent transcription of hormone-responsive genes with different transcription responses.[14,15] The activation of hormone receptors is unique in that the "phototransducer" is a readily diffusible small molecule. Therefore, the system only becomes transiently light-sensitive after addition of the caged agonist. These systems provide for unique time-dependent transcription responses.

The different ER subtypes (ERα and ERβ) can act upon genes bearing classic estrogen response elements (EREs), which are composed of a palindromic core sequence directly recognized by homodimeric ERs, or AP1 site promoters on which ER interacts indirectly through Jun and Fos.[16–20] Different combinations of subtypes and response elements have been shown to respond differently to a variety of ER ligands known as selective estrogen receptor modulators (SERMs). One of the most interesting examples is the partial antagonist tamoxifen, which has been found to antagonize estradiol (E2)–ERα on classic EREs, but to function as an agonist with ERβ on AP1 sites.[16]

Here, we report the first examples of photocaged SERMs, photocaged hydroxytamoxifen (NB-Htam) and photocaged guanidinotamoxifen (NB-Gtam), that can simultaneously mediate both light-activated gene expression and gene repression mediated by ERα and ERβ. These caged compounds act as neither agonists nor antagonists to ERα or ERβ until uncaged by the action of light. Such systems may constitute powerful new tools to create complex spatial and temporal patterns of expressed gene products by providing simultaneous activation and repression of different genes from the same stimulus and may further provide a means to selectively deliver SERMs to targeted tissues.

Results and Discussion

Chemistry

The well-established photochemical release of bioactive substrates, so-called photocaging, represents a powerful method

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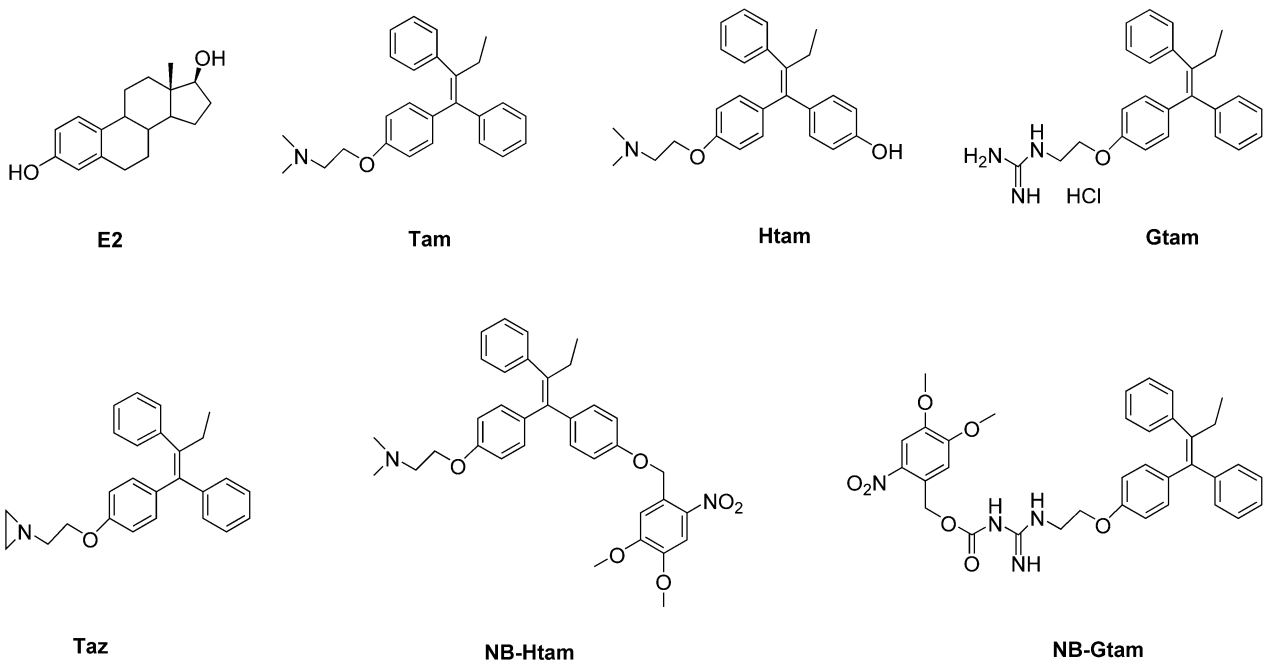


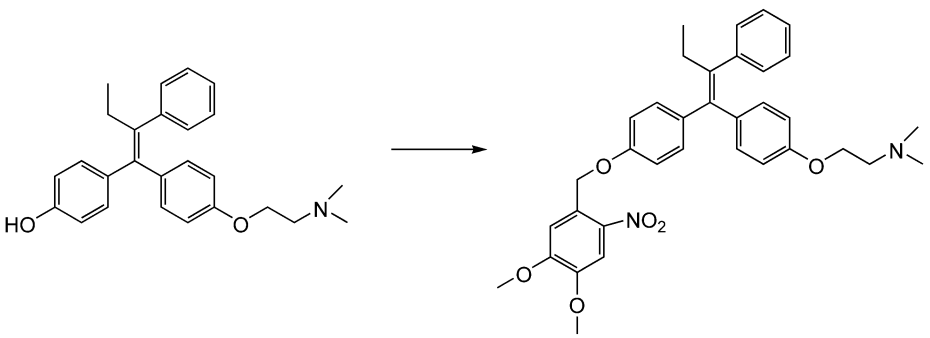
Scheme 1. *Chemical structures of ligands.*

to control biochemical or physiological responses in a spatially and temporally defined manner.[1] A photocaged compound should be biologically inert until irradiation causes the release of a biologically active compound. A variety of different photocaging groups (photolabile protecting groups) are commonly used, including groups that can be removed by using high-intensity visible light by the multiphoton effect.[21]

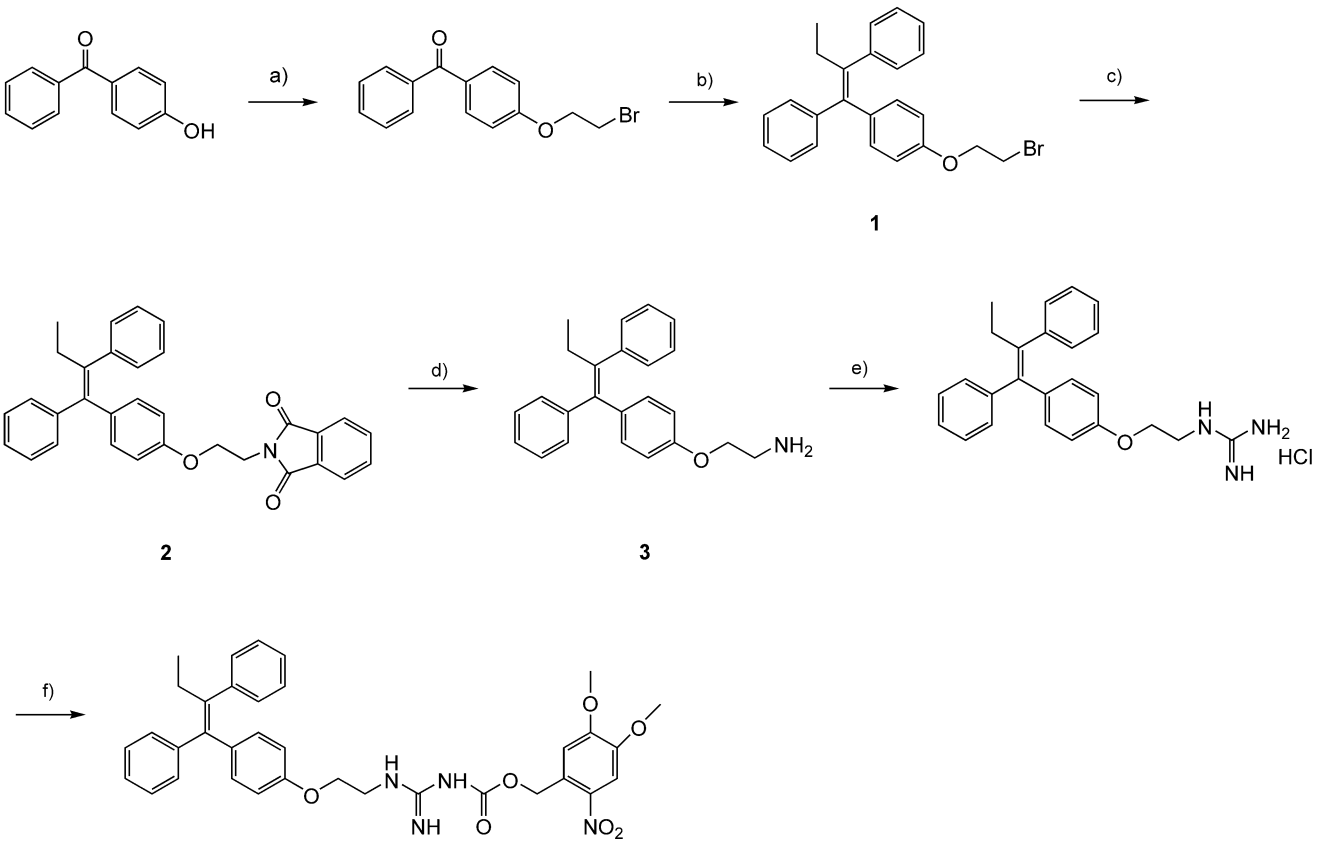
The nitroveratryl group (4,5-dimethoxy-2-nitrobenzyl group) is commonly used to protect alcohols and amines as ethers, carbonates, or carbamates and can be efficiently removed by mild irradiation with long-wavelength UV radiation (>345 nm). For synthetic and experimental convenience, we used the nitroveratryl group (NB) to photocage SERMs in this study; other photolabile protecting groups could presumably be used to cage these compounds depending on specific applications.

The structure of tamoxifen (Tam) does not afford an easy chemical handle with which to attach a photocaging group; however, 4-hydroxytamoxifen (Htam), a more potent ER antagonist, can be readily caged as its nitroveratryl ether. Similar to Tam, Htam has been shown to function at both classic EREs and AP1 sites (Scheme 1).[19] The phenolic 4-hydroxyl group on Htam is oriented in the ER ligand binding pocket in a similar manner to that of E2. The 3-nitroveratryl ether of E2 was effective in masking the agonist properties of E2;[14] therefore, it was reasoned that *O*-nitroveratryl would similarly cage Htam. Htam was therefore synthesized by following the method described by Gauthier et al. and alkylated with 4,5-dimethoxy-2-nitrobenzylbromide to afford the photocaged hydroxytamoxifen (NB-Htam) (Scheme 2).[22]

The binding of intracellularly released SERMs to ER must compete against the diffusion of free ligand out of the cell. For comparison, we also examined the effects of a caged SERM with a highly basic side-chain, which was expected to diffuse across membranes more slowly because a larger fraction of the ligand will be in its protonated, ionic form at physiological pH. Katzenellenbogen et al. reported a guanidine-substituted analogue of Tam, namely, guanidine tamoxifen (Gtam), which has similar antiestrogenic potency to Tam but which is more basic.[23] We also synthesized a photocaged form of Gtam to determine if this more basic ligand provided a different transactivation response in cell-based assays. Gtam required a different caging strategy because it lacks the phenol hydroxyl handle used to cage Htam. Therefore, we proposed to cage Gtam by modifying its guanidine side-chain (Scheme 3). A priori, it is not clear if modification of the gua-

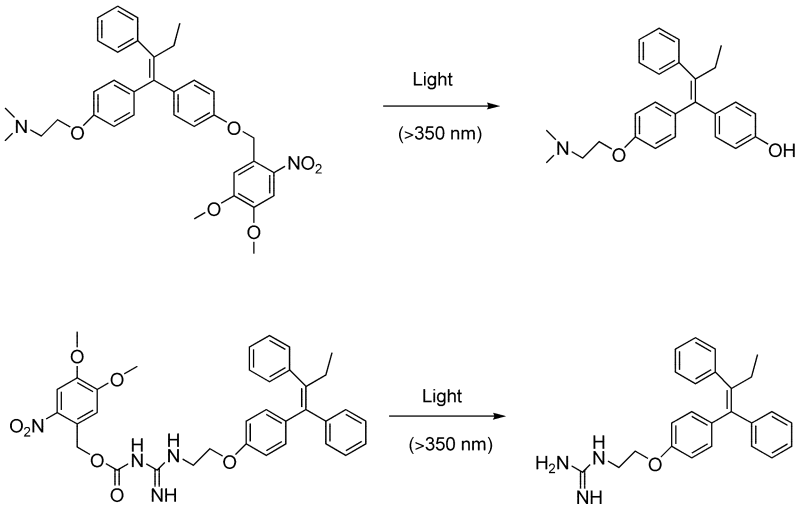


Scheme 2. *Synthesis of NB-Htam: NaH, 4,5-dimethoxy-2-nitrobenzylbromide, DMF, 0 °C to RT, 5 h.*



Scheme 3. *Reagents and reaction conditions for synthesis of NB-Gtam: a) 1,2-Dibromoethane, NaH, DMF, 80 °C, 5 h; b) $TiCl_4$, Zn, propiophenone, THF, reflux, 8 h; c) Potassium phthalimide, DMF, reflux, 2 h; d) Hydrazine hydrate, EtOH, reflux, 2 h; e) 1*H*-pyrazole-1-carboxamidine hydrochloride, DIEA, DMF, RT, 8 h; f) 6-Nitroveratryl chloroformate, 4 N NaOH, CH_2Cl_2, 0 °C, 10 h.*

nidine group would be sufficient to mask Gtam's antagonist properties. By analogy to the structure of the Tam–ER complex, one would expect the guanidine group of Gtam to lie on or near the surface or mouth of the ER binding pocket.[24] Although some potent ER antagonists which lack a basic side-chain are known, structure–activity (SAR) studies suggest that potent Tam analogues usually require a strongly basic side-chain.[23] Therefore, we synthesized the nitroveratryl carbamate (Nvoc) analogue of the guanidine of GTam (NB-Gtam), which was expected to have a significantly lower pK_a than GTam and may additionally provide steric encumbrance to Gtam's binding to ER. Ab initio calculations (RB3LYP6-31+(d,p)) of ethyl guanidine and ethylguanidinemethyl carbamate suggest that the gas-phase proton affinity of NB-Gtam should be lowered by 4.8 kcal mol^{-1} or 3.6 pK_a units compared to Gtam. The rate and efficiency of the photodeprotection of NB-Htam and NB-Gtam were determined by using an identical experimental set up to one used previously to activate ligands in cell culture.[14] The caged compound NB-Htam is converted to Htam upon irradiation with 85% conversion at a rate of 4.05×10^{-2} s^{-1} (Scheme 4). For purposes of comparison, (*Z*)tamoxifen (Tam) and (*Z*)tamoxifen aziridine (Taz) were also prepared according to the published procedures (Scheme 1).[25,26] Taz is known to irreversibly bind to ER through covalent bond formation by reaction of Cys530 of ERα with the aziridine of Taz.



Scheme 4. *Photocaged compounds release Htam and Gtam upon exposure to light.*

Human estrogen receptors (ERα and ERβ) are members of the superfamily of nuclear/steroid hormone receptors which activate the transcription of hormone-regulated genes in a ligand-dependent manner.[27,28] Upon binding estrogen, ERs undergo

a conformational change that enable them to bind to classic EREs and recruit upstream effectors. Whereas tamoxifen functions as an antagonist to ERs bound to classic EREs, it has recently been shown that Tam functions as an agonist to ERβ bound to AP1 response elements.[16,19,29]

Actions of caged SERMs with ERα and ERβ on classic EREs

Classic EREs are composed of two inverted hexanucleotide repeats to which ERs bind as homodimers.[17] Ligand-dependent transcription assays of ERα and ERβ with the caged SERMs NB-Htam and NB-Gtam as well as E2, Tam, Htam, and Gtam were performed by using reporter genes containing classic EREs. NB-Htam and NB-Gtam were evaluated for their ability to control exposure-dependent repression of ER-mediated transcription in HeLa cells transiently cotransfected with a luciferase reporter ERE-luc (classic vitellogen promoter), ER expression vectors (pSG5ERα or pSG5ERβ), and renilla luciferase control plasmid pRLCMV.[30] Cells were incubated in media containing 1.0 nM E2 and either 1 μM NB-HTam, NB-Gtam, Tam, Htam, or Gtam for 12 h. Cells were then either kept in the dark or briefly exposed to mild, long-wavelength UV radiation (>345 nm) for 120 s by using the apparatus described previously.[14] Previous studies have demonstrated that photocaged hormones can be uncaged intracellularly by using mild UV irradiation that does not otherwise adversely affect cell viability. Twenty four hours after irradiation, cells were evaluated for luciferase reporter gene activity by using a dual luciferase assay (Promega).

ERα and ERβ similarly activate transcription in response to E2 at classic EREs.[31] It has been shown that Tam, Htam, and Gtam function as potent antagonists, thereby repressing E2 activation of both ERα and ERβ (Figure 1). Importantly, while Tam, Htam, and Gtam are potent estrogen antagonists, the caged analogues NB-Htam and NB-Gtam function as neither agonists nor antagonists at concentrations of below 1 μM, thus demonstrating that our caging strategy was effective at masking the intrinsic biological activities of Tam and Gtam. Upon irradiation, NB-Htam and NB-Gtam are efficiently converted to potent ER antagonists and cause a 5- to 7-fold reduction in ER/E2 transcription similar to that observed by adding Gtam or Htam directly.

Much work has focused on the potency and properties of the Htam stereoisomers. It has been demonstrated that (*Z*)-Htam is an antagonist of ER, while (*E*)-Htam is a weak agonist.[32,33] However, it has also been shown that (*Z*)-Htam and (*E*)-Htam will rapidly isomerize in vitro and in vivo, and the mixture of isomers has overall antagonist activity, since the (*Z*)-Htam binds to ER much more tightly than (*E*)-Htam.[33,34] Therefore, though NB-Htam may be photoisomerized upon irradiation, uncaged Htam shows full antagonist activity in culture.

Diffusion experiments

The use of caged ligands to regulate gene expression in a spatially and temporally defined manner can be limited by the diffusion of the photoreleased ligand into surrounding unexposed tissue or media.[6,14] Such diffusion would be expected

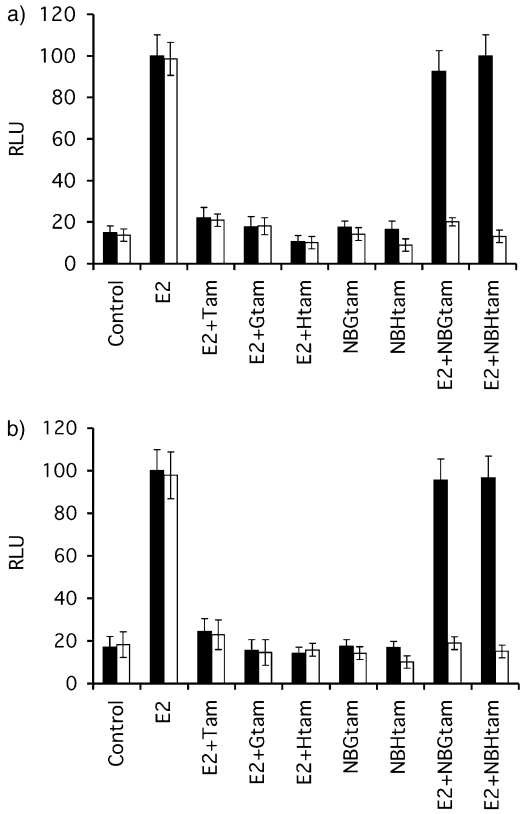


Figure 1. *Ligand-dependent activation of ERE reporter gene expression in HeLa cells with irradiation (white bars) or without irradiation (black). HeLa cells were transfected with an ERE-regulated luciferase reporter plasmid and an expression vector for ERs. a) ERα; b) ERβ. Transfected cells were treated with one or two ligands as indicated (E2, 1 nM; Tam, 1 μM; Htam, 1 μM; Gtam, 1 μM; NB-Htam, 1 μM; and NB-Gtam, 1 μM) or an ethyl alcohol (EtOH) vehicle (control). Error bars show deviations between wells from six representative transfection. RLU = relative light units. Data are mean ± SEM (standard error of the mean) of six independent experiments run in triplicate.*

to reduce the duration and spatial resolution that can be achieved by this approach. Ligands with structures that diffuse more slowly across membranes might be expected to provide a longer duration response than more rapidly diffusing ligands. Barring any active diffusion mechanisms, transmembrane diffusion of basic ligands such as Tam and Gtam across membranes will be impeded by an amount related to the fraction of ligand in its charged protonated state at physiological pH. Owing to its dimethylamino side-chain ($pK_a \approx 10.8$), Tam is expected to diffuse across membranes approximately 800 times ($10^{2.9}$) faster than Gtam whose guanidine side-chain has a pK_a of approximately 13.7.[23]

We compared the duration of repression response observed with Tam and Gtam in our cellular assays by using a classic ERE reporter present only intracellularly and allowed to freely diffuse out of the cells into the surrounding media. For comparison, we also used Taz, which irreversibly binds to ER through formation of a covalent bond.[26] In these studies, HeLa cells, transiently transfected with pSG5ERα, reporter ERE-luc, and control, were allowed to incubate in the presence of 10 nM of E2 and 1 μM of either Tam, Gtam, or Taz. After 2 h of preincu-

bation, the media were exchanged with fresh media containing the same concentration of E2 (10 nM) but without antagonist, thus allowing the intracellular concentration of antagonist to dilute through diffusion, whereas the intracellular concentration of E2 remained unchanged. Reporter gene product formation was measured at various time intervals after media exchange to follow the loss of antagonist-induced repression (Figure 2). Initially, Gtam and Tam cause similar amounts of repression; however, reporter gene product formation is recovered more quickly with Tam than with Gtam or Taz. Similar results are obtained when E2 and antagonists are preincubated for 5 h instead of 2 h (data not shown). Though Gtam is approximately 4 pK_a units more basic than Tam, transcription is recovered for both ligands at similar rates (6.4 h^{-1} versus 5.6 h^{-1}) (Figure 2). These results show that the duration of repression response is not substantially influenced by the basicity of the ligand. They further suggest that transmembrane diffusion controlled by altering the charge state of the ligand does not significantly alter the duration of transrepression response and may reflect differences in the relative binding affinity (RBA) of Tam and Gtam for ERα (Gtam RBA = 5.3; Tam RBA = 2.1).[23]

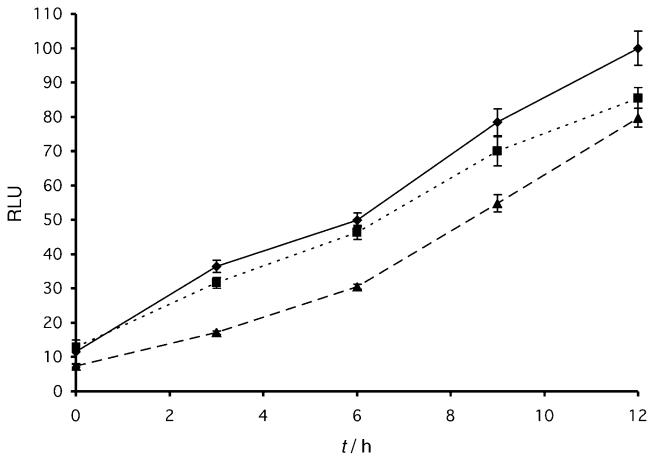


Figure 2. *Time-dependent ERα action at an ERE response element in HeLa cells. HeLa cells were transfected with an ERE reporter plasmid and an ERα expression plasmid. Transfected cells were treated with E2, 10 nM; Tam, 1 μM (◆); Gtam, 1 μM (■); Taz, 1 μM (▲) for 2 h prior to exchanging to ligand-free media. RLU = relative light units.*

Cells treated with Taz, an irreversible ER antagonist, similarly recovered almost complete activity in 12 h but at a significantly slower rate (3.84 h^{-1}) than observed with Tam or Gtam. These results strongly suggest that the duration of the antagonist action of Tam and Gtam is limited by the ligands' off-rates from the receptor as opposed to their transmembrane diffusion or turnover of the ligand–receptor complex. This also sets a practical limitation on the duration of transient photoinduced repression response, which is ultimately limited by the resynthesis of new ER. These findings additionally provide insights into the dynamic nature of SERM action.

SERM-activated transcription with ERα(G400V/M539A/L540A) and ERα(G400V/L543A/L544A) on classic EREs

The successful design of our caged SERMs NB-Htam and NB-Gtam to function as light-activated antagonists prompted us to further investigate their potential role as light-activated hormone receptor agonists. Recently, Wahli reported a double mutant of mouse ER that provided a transactivation response to the otherwise antagonist Htam.[35] The ER stimulates transcription of target genes by means of its two transcriptional activation domains, namely, AF-1 in the N-terminal part of the receptor, and AF-2 in its ligand binding domain. Mutagenesis and structural studies have shown that amino acids 538–552 (helix 12) are critical for the ability of ER to interact with its effector proteins. It is believed that agonist binding induces conformational changes in AF-2 that create a hydrophobic surface to which co-activators can bind. AF-2 activity is dependent upon a putative amphipathic α helix between residues 538 and 552 in the mouse ER. In particular, it has been reported that the mouse ER mutants *m*ER(L543A/L544A) and *m*ER(M547A/L548A) change the properties of antiestrogens to full agonist with Htam.[35] We proposed that the analogous mutations to *human* ERα might similarly respond to Htam as an agonist, thereby potentially allowing us to use NB-Htam as a light-activated transcriptional activator. The two human ER mutants ERα(G400V/L539A/L540A) and ERα(G400V/M543A/L544A) were generated by site-directed mutagenesis (Quickchange, Strategene). Their ligand-dependent transcriptional activities were evaluated by cell-based assays. Substitution of either one pair of hydrophobic residues (L539A/L540A or M543A/L544A) resulted in a markedly lower basal activity in the absence of hormone as compared to the wild-type ER. E2 had only a weak effect on the transcriptional activity of these two mutants; however, Htam was not observed to be an agonist of ER but rather still functioned as an antagonist of these mutant receptors (Figure 3). The activation of ERα mutants by antiestrogenic compounds previously reported with mouse ER mutants was not observed with the analogous human ER variants.

Caged SERMs mediate light-activated transcription of AP1-responsive genes with ERα and ERβ

The two subtypes of human estrogen receptors, ERα and ERβ, regulate transcription at an AP1 response element differently in response to E2 and the antiestrogens, Tam and Htam.[16,18,19,36] ER associates with AP1 in complex with either heterodimers of members of the Fos and Jun families of proteins or with homodimers of members of the Jun family of proteins.[37,38] Here, we evaluated the ability of our photocaged ligands (NB-Htam and NB-Gtam) to induce AP1-driven luciferase reporter gene expression in HeLa cells transiently transfected with either ERα or ERβ.

Similar to Tam and Htam, we show that Gtam also affects ligand-dependent transactivation response at AP1 sites. A comparison of the activation profiles for each ligand at an AP1 site with ERα and ERβ shows that the antiestrogens, Tam, Htam, and Gtam, can fully activate gene expression through ERβ,

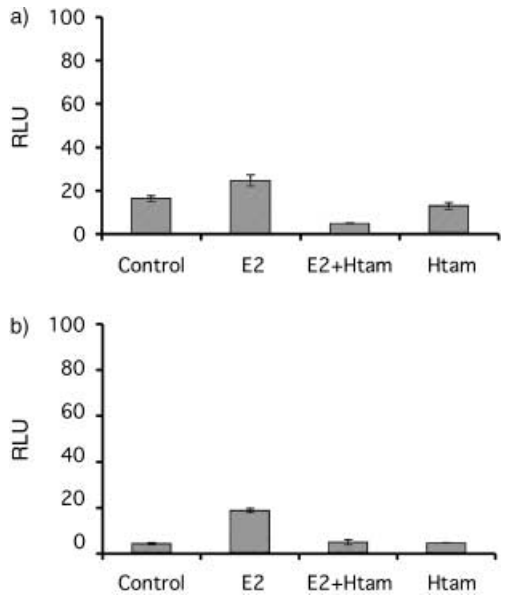


Figure 3. *Ligand-dependent ERα and mutants action at an ERE in HeLa cells. HeLa cells were transfected with an ERE-regulated luciferase reporter plasmid and an ERα expression plasmid and treated with 100 nM of E2 or 0.5 μM of Htam, respectively. a) ERα(G400V/L539A/L540A); b) ERα(G400V/M543A/L544A). Data are mean ± SEM of four independent experiments run in triplicate. RLU = relative light units.*

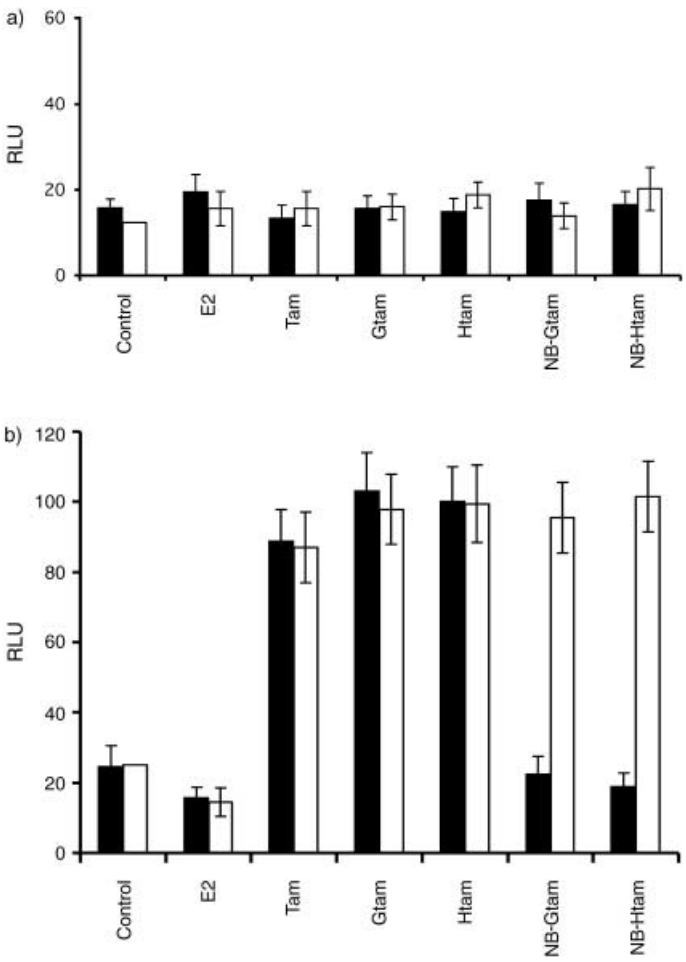


Figure 4. *Ligand-dependent ER action at an AP1-responsive reporter in HeLa cells in the dark (black) or light (white). HeLa cells were transfected with an API reporter plasmid and ERs expression plasmid and treated with ligands as indicated. a) ERα; b) ERβ. Ligand concentrations were E2, 100 nM; Tam, 1 μM; Htam, 1 μM; Gtam, 1 μM; NB-Htam, 1 μM; and NB-Gtam, 1 μM. Data are mean ± SEM of six independent experiments run in triplicate. RLU = Relative light units.*

whereas no activation was observed with ERα (Figure 4). In the absence of light, the caged compounds NB-Htam and NB-Gtam do not activate AP1-driven gene expression, but upon light exposure both NB-Gtam and NB-Htam become essentially full agonists of ERβ. These findings demonstrate the unique ability of NB-Htam and NB-Gtam to simultaneously mediate photodependent gene activation and repression of two different gene products, thus providing a novel method to photoinitiate complex gene expression patterns in tissue.

Conclusion

Photoactivated SERMs provide a unique method to spatially and temporally control gene expression in multicellular systems. The photocaged SERMs NB-Tam and NB-Gtam provide a robust exposure-dependent antagonism of ER-mediated transcription on ERE-responsive reporters. These antagonist effects are only transient when ligand is allowed to dilute out of the cell into media lacking SERM. Comparison of the time required to release the antagonist actions of intracellular Tam and Gtam on ER-mediated transcription at classic EREs suggest that the duration of antagonist effect is likely limited by the ligand's off-rate from the receptor and not the rate of transmembrane diffusion of free ligand out of the cell or degradation of the receptor. Under similar conditions, the covalent-binding antagonist Taz establishes an upper limit on the duration of photoinduced transrepression response that is ultimately controlled by the rate of resynthesis of the receptor.

The opposing actions of SERMs at classic EREs and AP1 enables caged SERMs to simultaneously mediate both photoactivated gene transcription and repression. In combination with earlier studies which have shown that hormone receptors can be re-engineered to selectively respond to synthetic hormone analogues that do not activate endogenous receptors, nuclear-receptor-mediated light activation of gene transcription/repression may offer a unique tool for studying the actions of patterned gene expression.

Experimental Section

General: All reagents were purchased from Aldrich Chemical Co. All reactions were performed in flame-dried glassware under a positive atmosphere of dry N_2. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone prior to use. All extracts were dried over $MgSO_4$, and solvents were removed by rotary evaporation under reduced pressure. Column chromatography was carried out on silica gel (230–400 mesh) (ICN). Photocaged compounds were synthesized under reduced light and kept in the dark at −20 °C. ^{1}H NMR spectra were recorded at 400 MHz, and ^{13}C NMR spectra were recorded at 100 MHz. Mass spectra were measured by the University of Delaware Mass Spectrometry Facility. HeLa

cells were obtained from ATTC (American Type Tissue Collection) and maintained at the University of Delaware Cell Culture Core Facility. Cell culture media were purchased from Mediatech, Inc (Herndon, VA). Transactivation response assays were performed by using the Dual-Luciferase Reporter Assay System (Promega #E1960) according to the manufacturer's protocol. Active protein one (AP1)-responsive luciferase reporter was generously provided by Prof. Thomas S. Scanlan (UCSF).[16]

Plasmid constructions: Plasmids pSG5-hERα(G400V/M539A/L540A) and pSG5-hERα(G400V/L543A/L544A) were constructed from the parent plasmid pSG5-hERα[30] by oligonucleotide-directed mutagenesis performed using Quickchange (Stratagene) and the following oligonucleotides: GTGCCCCTCTATGACGCGGCGCTGGAGATGCTGGAC, GTCCAGCATCTCCAGCGCCGCGTCATAGAGGGGCAC, CCTGCTGCTGGAGGCGGCGGACGCCCACCG, and CGGTGGGCGTCCGCCGCCTCCAGCAGCAGG. The identity of all constructs was confirmed by restriction enzyme mapping and DNA sequence analysis.

Cell culture, transfection, and luciferase assay: HeLa cells were seeded at a density of 40000 cells per well in a 24-well culture plate and grown in phenol-red-free Dulbecco's modified Eagle's medium (DMEM) with supplemented 10% charcoal/resin-stripped fetal bovine serum (FBS, Summit Biotechnology, lot FA0727) 24 h prior to transfection. All cells were routinely incubated in a humidified 5% CO_2-containing atmosphere at 37 °C.

Transfections were performed by the calcium phosphate coprecipitation method with 0.03 μg pRL basic (or AP1), 0.14 μg ERE-Luc$^+$, and the optimal amount of the receptor expression vector. The optimal receptor plasmid concentration for maximal ligand activation was determined for each receptor and was found to 0.08 μg plasmid per well for mutants, but only 0.02 μg expression plasmid per well for hERα and hERβ. Six hours after the transfection, the media were removed and replaced with DMEM + 10% charcoal-resin-stripped FBS containing appropriate concentrations of ligand. The cells were allowed to incubate with the new media for 30 h before harvesting by passive lysis.

Luciferase assays were performed by dual luciferase reporter assay (Promega) by using a Dynex luminometer according to the manufacturer's protocol. Activity is reported as relative light units (RLU) determined as the ratio of the firefly luminescence divided by the luminescence of the renilla luciferase control. The RLU values of different experiments are normalized such that 100 RLU corresponds to the full inducible activity of ER with E2 (10 nM) for classic EREs and ERβ with 1 μM Htam for the AP1-responsive reporter.

UV exposure of cells with photocaged ligands: HeLa cells were transiently transfected with respective receptor, reporter, and control. The cells were allowed to incubate for 12 h in the presence of photocaged ligands. The samples were then irradiated with long-wavelength UV radiation ($>$345 nm) through the culture plate lid for 120 s, as described previously.[14] The cells were allowed to incubate for another 24 h before harvesting by passive lysis.

Measurement of intracellular ligand diffusion: To determine the intracellular diffusion of ligands, time-dependent ERα action at an ERE response element in HeLa cells was measured. HeLa cells were transfected with an ERE reporter plasmid and an ERα expression plasmid. Transfected cells were treated with E2 (10 nM) and Tam (1 μM), Htam (1 μM), Gtam (1 μM), or Taz (1 μM). Cells were allowed to incubate for 2 h and 5 h in media containing E2 + SERM before exchanging to SERM-free media. Media were exchanged by first removing the media from the cells, immediately washing the cells with PBS buffer, and adding new media containing 10 nM of E2. The cells were allowed to incubate for 0–12 h before harvesting by passive lysis.

HPLC analyses and kinetics study of the photolysis of NB-Htam: All caged samples tested in culture were analyzed by analytical HPLC. To determine the kinetics of the photolysis of NB-Htam, the HPLC method was utilized for determination of the rate constant of photodeprotection of NB-Htam. A reaction solution of NB-Htam in MeOH containing 1.0 mM of NB-Htam was prepared for irradiation. Nine separate UV irradiation exposure times were employed in triplicate (120 μL total volume): 0 s, 5 s, 10 s, 20 s, 40 s, 80 s, 120 s, 240 s, and 480 s. The samples were irradiated through a glass plate and polystyrene culture plate lid, as described previously.[14] The loss of NB-Htam and the production of Htam were quantified by HPLC by using a Shimadzu FCV-10AC pump, SCL-10A UV/Vis detector (Shimadzu Scientific Instruments, Inc, Columbia, MD, USA) with an Alltech Econosil C18, 5 μm , 250 mm × 4.6 mm i.d. column (Alltech Associates, Inc., Deerfield, IL, USA), and a mobile phase composed of methanol (50%), acetonitrile (31%), water (18.9%), and trifluoroacetic acid (0.1%).

Synthesis

(*E*)- and (*Z*)-4-hydroxytamoxifen: (*E*)- and (*Z*)-Htam (4-(1-(4-(2-dimethylaminoethoxy)phenyl)-2-phenylbut-1-enyl)phenol) were prepared according to reported methods.[22]

(*Z*)-(2-(4-(1-(4-(4,5-Dimethoxy-2-nitrobenzyloxy)phenyl)-2-phenylbut-1-enyl)phenoxy)ethyl)dimethylamine (NB-Htam): A solution of (*Z*)-4-hydroxytamoxifen (40 mg, 0.1 mmol) in *N*,*N*-dimethylformamide (DMF) (3 mL) was added to a rapidly stirred suspension of NaH (5 mg, 60% in oil, prewashed with hexanes) in DMF (1 mL). The resulting solution was stirred at 0 °C for 45 min before a solution of 4,5-dimethoxy-2-nitrobenzylbromide (42 mg, 0.15 mmol) in DMF (1 mL) was added. The reaction mixture was stirred at room temperature under nitrogen for 5 h, poured into ice-cold H_2O (20 mL), and then extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by chromatography (CH_2Cl_2/methanol, 19:1) to afford (*Z*)-NB-Htam (20 mg, 46%) and the major side-product *N*,*O*-dinitrobenzylhydroxytamoxifen (DNB-Htam, 30 mg, 40%) as a light yellow solid.

NB-Htam: ^{1}H NMR ($CDCl_3$): $\delta = 0.93$ (t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.31 (s, 6H; $N(CH_3)_2$), 2.49 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 2.68 (t, $J = 5.5$ Hz, 2H; CH_2CH_2N), 3.96(t, $J = 5.5$ Hz, 2H; OCH_2CH_2), 3.97 (s, 3H; $ArOCH_3$), 3.98 (s, 3H; $ArOCH_3$), 5.52 (s, 2H; $ArCH_2O$), 6.56 (d, $J =$ 8.7 Hz, 2H; Ar*H* of tamoxifen), 6.76 (d, $J = 8.7$ Hz, 2H; Ar*H* of tamoxifen), 6.99 (d, $J = 8.6$ Hz, 2H; Ar*H* of tamoxifen), 7.09–7.20 (m, 7H; Ar*H* of tamoxifen), 7.39 (s, 1H; Ar*H* of nitrobenzene), 7.79 ppm (s, 1H; Ar*H* of nitrobenzene); ^{13}C NMR ($CHCl_3$): $\delta = 13.85$, 29.24, 45.90, 56.61, 58.31, 65.63, 67.30, 108.16, 109.65, 113.52, 114.81, 126.16, 128.06, 129.78, 129.87, 131.00, 132.08, 135.94, 137.43, 137.75, 139.02, 141.44, 142.66, 147.98, 154.12, 156.95 ppm; HRMS: *m*/*z* calcd for $C_{35}H_{39}N_2O_6$ $[M+1]^+$: 583.2808; found: 583.2802.

DNB-Htam: ^{1}H NMR ($CDCl_3$): $\delta = 0.93$ (t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.49 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 3.29 (s, 6H; $N(CH_3)_2$), 3.98 (s, 6H; $ArOCH_3$), 3.99 (s, 3H; $ArOCH_3$), 4.12 (s, 3H; $ArOCH_3$), 4.23 (t, $J =$ 5.5 Hz, 2H; OCH_2CH_2), 4.40 (t, $J = 5.5$ Hz, 2H; $CH_2CH_2N^+$), 5.51 (s, 2H; $ArCH_2O$), 5.71 (s, 2H; $ArCH_2N^+$), 6.56 (d, $J = 8.7$ Hz, 2H; Ar*H* of tamoxifen), 6.81 (d, $J = 8.7$ Hz, 2H; Ar*H* of tamoxifen), 7.00 (d, $J =$ 8.6 Hz, 2H; Ar*H* of tamoxifen), 7.09–7.18 (m, 7H; Ar*H* of tamoxifen), 7.39 (s, 1H; Ar*H* of nitrobenzene 1), 7.70 (s, 1H; Ar*H* of nitrobenzene 2), 7.78 (s, 1H; Ar*H* of nitrobenzene 1), 8.19 ppm (s, 1H; Ar*H* of nitrobenzene 2); ^{13}C NMR ($CDCl_3$): $\delta = 16.82$, 17.21, 22.87, 51.06,

56.24, 56.34, 59.18, 61.98, 63.65, 64.48, 67.29, 108.09, 109.05, 109.64, 114.24, 115.80, 119.50, 126.36, 127.96, 129.80, 130.67, 132.27, 136.25, 136.96, 137.22, 137.76, 139.20, 142.28, 142.36, 142.77, 148.00, 150.83, 153.83, 154.10, 154.85, 157.08 ppm.

(*E*)-(2-(4-(1-(4-(4,5-Dimethoxy-2-nitrobenzyloxy)phenyl)-2-phenylbut-1-enyl)phenoxy)ethyl)dimethylamine ((*E*)-NB-Htam): (*E*)-NB-Htam was made by following the same protocol used to make (*Z*)-NB-Htam except starting from (*E*)-4-hydroxytamoxifen (40 mg, 0.1 mmol). ^{1}H NMR ($CDCl_3$): $\delta = 0.93$ (t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.35 (s, 6H; $N(CH_3)_2$), 2.50 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 2.75 (t, $J = 5.5$ Hz, 2H; CH_2CH_2N), 3.88 (s, 3H; $ArOCH_3$), 3.94 (s, 3H; $ArOCH_3$), 4.08(t, $J = 5.5$ Hz, 2H; OCH_2CH_2), 5.36 (s, 2H; $ArCH_2O$), 6.65 (d, $J = 8.7$ Hz, 2H; Ar*H* of tamoxifen), 6.81 (d, $J = 8.7$ Hz, 2H; Ar*H* of tamoxifen), 6.90 (d, $J = 8.6$ Hz, 2H; Ar*H* of tamoxifen), 7.08–7.15 (m, 7H; Ar*H* of tamoxifen), 7.24 (s, 1H; Ar*H* of nitrobenzene), 7.73 ppm (s, 1H; Ar*H* of nitrobenzene); ^{13}C NMR ($CDCl_3$): $\delta = 13.78$, 29.19, 46.08, 56.50, 58.49, 66.00, 66.91, 108.01, 109.47, 113.95, 114.21, 126.109, 127.96, 129.80, 130.67, 132.26, 136.25, 136.91, 137.79, 139.01, 141.52, 142.71, 147.79, 153.96, 156.00, 157.72 ppm; HRMS: *m/z* calcd for $C_{35}H_{38}N_2O_6$; 582.2730; found: 582.2714.

(*Z*)-1-(4-(2-Bromoethoxy)phenyl)-1,2-diphenylbut-1-ene (1): The title compound was prepared according to the literature procedure from 4-(2-bromoethoxy)benzophenone and propiophenone by McMurry reaction and purified by recrystallization from propan-2-ol.[25]

(*Z*)-1-(4-(2-Phthalimidoethoxy)phenyl)-1,2-diphenylbut-1-ene (2): Potassium phthalimide (0.18 g, 0.7 mmol) was added to a solution of (*Z*)-1-(4-(2-bromoethoxy)phenyl)-1,2-diphenylbut-1-ene (**1**) (0.26 g, 0.6 mmol) in dry DMF (5 mL). The solution was heated at reflux for 2 h and then cooled to room temperature. Water (10 mL) was added, and the reaction mixture was extracted with CH_2Cl_2 (3×20 mL). The combined organic extracts were washed with water (2×20 mL), brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was recrystallized from EtOH to afford the title compound (0.25 g, yield 83%) as colorless crystals. ^{1}H NMR ($CDCl_3$): $\delta = 0.91$ (t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.44 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 2.76 (t, $J = 5.5$ Hz, 2H; CH_2CH_2N), 4.07 (t, $J = 5.5$ Hz, 2H; OCH_2CH_2), 6.51 (d, $J = 8.7$ Hz, 2H; Ar*H*), 6.74 (d, $J = 8.7$ Hz, 2H; Ar*H*), 7.10–7.32 (m, 10H; Ar*H*), 7.70 (t, $J = 5.4$, 3.2 Hz, 2H; Ar*H* of phthalimide), 7.84 ppm (t, $J = 5.4$, 3.2 Hz, 2H; Ar*H* of phthalimide); ^{13}C NMR ($CDCl_3$): $\delta = 13.78$, 29.20, 37.51, 64.60, 113.59, 123.53, 126.24, 126.71, 128.06, 128.28, 129.63, 129.85, 132.07, 132.21, 134.20, 136.11, 141.61, 142.52, 156.41, 168.32 ppm; HRMS: *m/z* calcd for $C_{32}H_{27}NO_3$: 473.1991; found: 473.1976.

(*Z*)-1-(4-(2-Aminoethoxy)phenyl)-1,2-diphenylbut-1-ene (3): Hydrazine hydrate (0.2 mL) was added to a solution of (*Z*)-1-(4-(2-phthalimidoethoxy)phenyl)-1,2-diphenylbut-1-ene (**2**) (0.25 g, 0.5 mmol) in EtOH (10 mL). The solution was heated at reflux for 2 h, cooled to room temperature, and then acidified to pH 2–3 with 2 M HCl. The resulting precipitate was removed by filtration, and the filtrate was partially concentrated to remove the ethanol. The resulting aqueous solution was made alkaline (pH 8–9) by the addition of 2 M NaOH, and washed with EtOAc (3×20 mL). The combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by chromatography (CH_2Cl_2/MeOH, 9:1) to afford the desired product (0.15 g, yield 82.5%) as a colorless oil. ^{1}H NMR ($CDCl_3$): $\delta = 0.92$ (t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.46 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 2.99 (t, $J = 5.5$ Hz, 2H; CH_2CH_2N), 3.85 (t, $J = 5.5$ Hz, 2H; OCH_2CH_2), 6.54 (d, $J = 8.7$ Hz, 2H; Ar*H*), 6.76 (d, $J = 8.7$ Hz, 2H; Ar*H*), 7.11–7.34 ppm (m, 10H; Ar*H*); ^{13}C NMR ($CDCl_3$): $\delta = 13.82$, 29.22, 41.76, 46.40, 64.91, 71.60, 113.47, 126.21, 126.72, 128.08, 128.30, 129.66, 129.89, 132.12, 138.36, 141.53, 143.07, 143.99, 156.97 ppm; HRMS: *m/z* calcd for $C_{24}H_{25}NO$: 343.1936; found: 343.1923.

(*Z*)-1-(4-(2-*N*-Guanidinoethoxy)phenyl)-1,2-diphenylbut-1-ene hydrochloride (tamoxifen guanidine hydrochloride, Gtam): 1*H*-Pyrazole-1-carboxamidine hydrochloride (60 mg, 0.4 mmol) and *N*,*N*-diisopropylethylamine (DIEA, 0.1 mL) were added to a solution of 1-(4-(2-aminoethoxy)phenyl)-1,2-diphenylbut-1-ene (**3**) (0.15 g, 0.4 mmol) in DMF (1 mL). The resulting solution was stirred at room temperature under nitrogen overnight and then concentrated under reduced pressure. The residue was purified by chromatography (CH_2Cl_2/MeOH, 9:1) to afford Gtam (0.14 g, 82.5% yield) as colorless crystals. ^{1}H NMR ($CDCl_3$): $\delta = 0.92$ t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.45 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 3.35 (t, $J = 5.5$ Hz, 2H; CH_2CH_2N), 3.77 (t, $J = 5.5$ Hz, 2H; OCH_2CH_2), 6.44 (d, $J = 8.7$ Hz, 2H; Ar*H*), 6.75 (d, $J = 8.7$ Hz, 2H; Ar*H*), 7.08–7.31 (m, 12H; Ar*H*, NH), 7.95 (br s, 1H), 9.05 ppm (s, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 13.73$, 29.20, 41.71, 50.52, 67.73, 113.64, 126.36, 126.77, 128.11, 128.32, 129.54, 129.77, 132.15, 136.84, 138.01, 141.97, 142.32, 143.69, 155.52, 158.80 ppm; HRMS: *m/z* calcd for $C_{25}H_{27}N_3O$: 385.2154; found: 386.2230 ($M+H^+$).

***N*-(2-(4-(1,2-Diphenylbut-1-enyl)phenoxy)ethyl)-*N*′-(nitroveratryloxycarbonyl)guanidine (NB-Gtam)**: Aqueous NaOH (4 M, 0.1 mL) was added to a solution of Gtam (20 mg, 0.05 mmol) in CH_2Cl_2 (1 mL). The mixture was cooled to 0°C in an ice bath in the dark, and a solution of 6-nitroveratryl chloroformate (15 mg, 0.05 mmol) in CH_2Cl_2 (0.5 mL) was added. The reaction was stirred at 0°C overnight in the dark and then allowed to warm to room temperature. The aqueous layer was extracted with CH_2Cl_2 (3×2 mL), and the combined organic extracts were washed with 0.1 M HCl (5 mL), water (3×3 mL), brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by chromatography (hexane/EtOAc, 6:4) to afford NB-Gtam (22 mg, 74%). ^{1}H NMR ($CDCl_3$): $\delta =$ 0.83 (t, $J = 7.4$ Hz, 3H; CH_2CH_3), 2.35 (q, $J = 7.4$ Hz, 2H; CH_2CH_3), 3.88 (s, 3H; $ArOCH_3$), 3.90 (s, 3H; $ArOCH_3$), 3.94 (t, $J = 5.5$ Hz, 2H; CH_2CH_2N), 4.00 (t, $J = 5.5$ Hz, 2H; OCH_2CH_2), 5.53 (s, 2H; $ArCH_2O$), 6.40 (d, $J = 8.8$ Hz, 2H; Ar*H*), 6.67 (d, $J = 8.8$ Hz, 2H; Ar*H*), 7.09–7.34 (m, 12H; Ar*H*), 8.41 (d, 2H; NH), 9.23 (br s, 1H; NH), 9.41 ppm (br s, 1H; NH); ^{13}C NMR ($CDCl_3$): $\delta = 11.85$, 29.90, 44.29, 56.79, 57.02, 64.32, 65.27, 66.39, 107.40, 109.77, 125.54, 127.41, 129.38, 129.62, 136.34, 136.87, 139.58, 141.87, 142.51, 143.80, 148.02, 148.83, 153.36, 153.91, 153.93, 155.61, 156.33, 160.58, 163.24 ppm; HRMS: *m/z* calcd for $C_{35}H_{36}N_4O_7$: 624.2584; found: 624.2576.

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