

## Molecular design, chemical synthesis, and biological evaluation of agents that selectively photo-degrade the transcription factor estrogen receptor- $\alpha$ †

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2-Phenylquinoline (**1**) degraded proteins under photo-irradiation with long-wavelength UV light without additives and under neutral conditions. We designed and synthesized a 2-phenylquinoline-estrogen receptor- $\alpha$  (ER- $\alpha$ ) agonist (hybrid **2**) and a 2-phenylquinoline-ER- $\alpha$  antagonist (hybrid **3**) containing estradiol and 4-hydroxytamoxifen moieties, respectively. These 2-phenylquinoline hybrids effectively and selectively photo-degraded the target transcription factor, ER- $\alpha$ , which has a high affinity for estradiol and 4-hydroxytamoxifen. Target-selective photo-degradation was examined in both glass vessels and MCF-7 breast cancer cells, which are dependent upon ER- $\alpha$  for growth. In addition, 2-phenylquinoline-estradiol hybrid **2** functioned as an agonist of ER- $\alpha$  and promoted growth of MCF-7 in the absence of photo-irradiation, while it inhibited the growth of MCF-7 cells upon photo-irradiation due to the photo-degradation of ER- $\alpha$ . In contrast, 2-phenylquinoline-4-hydroxytamoxifen hybrid **3** inhibited growth of MCF-7 cells in the absence of photo-irradiation due to the antagonist effect of the 4-hydroxytamoxifen moiety against ER- $\alpha$ , and upon photo-irradiation significantly inhibited cell growth due to the dual antagonist effect of the 4-hydroxytamoxifen moiety and photo-degradation of ER- $\alpha$ .

### Introduction

The development of novel methods for selectively controlling specific protein functions is of considerable importance in the fields of chemistry, biology, and medicine. Protein degradation plays a central role in many cellular functions. For example, misfolded and damaged proteins are degraded and removed from cells to avoid toxicity, and the cellular concentration of regulatory proteins is maintained at optimal levels by degradation. In this context, much attention has been given to development of an organic photo-activatable agent that can degrade proteins<sup>1</sup> upon irradiation with a specific wavelength of light under mild conditions and without any additives (such as metals or reducing agents).

One of the most important applications of such selective photo-degrading agents is in the field of photodynamic therapy (PDT). PDT is used to treat diseases characterized by neoplastic growth, including various cancers, age-related macular degeneration, and actinic keratosis.<sup>2</sup> Cell death is induced by the activity of a reactive oxygen species (ROS) such as singlet oxygen  $^1\text{O}_2$ ,

generally produced by the photo-excitation of a light-activated photo-sensitizer molecule. One of the most important benefits of PDT compared with other therapies is the ease of space and time control for treatment. However, one of the drawbacks of PDT is its potential for serious side effects, such as systemic toxicity and photosensitivity resulting from delocalization of a photo-sensitizer lacking molecular-targeted selectivity. The use of light-activated and molecular-targeted (LAMTA) molecules may minimize the potential side effects associated with PDT by preventing delocalization of photo-sensitizers, thereby providing greater advantages than are achievable with conventional agents. In addition, LAMTA molecules could potentially be a very useful new class of pinpoint tools in a wide range of applications in the life sciences. However, to date there have been no reports of methods employing light-activated agents for selective degradation of target proteins. In this paper, we report the target-selective degradation of a protein induced by a light-activated small organic molecule.<sup>3</sup>

We found that 2-phenylquinoline degrades proteins in solution under long-wavelength UV photo-irradiation without additives and under neutral conditions. Two 2-phenylquinoline synthetic hybrids, 2-phenylquinoline-estradiol and 2-phenylquinoline-4-hydroxytamoxifen, were shown to effectively and selectively photo-degrade recombinant estrogen receptor- $\alpha$  (ER- $\alpha$ ), a key transcription factor involved in the growth of MCF-7 breast cancer cells. The target-selective degradation of ER- $\alpha$  led to the inhibition of MCF-7 cell growth. To the best of our knowledge, this is the first successful demonstration of target-selective degradation of a

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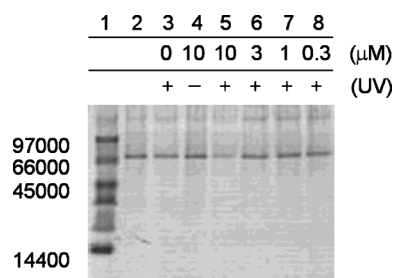
protein in glass vessels and in cell culture by light switching under neutral conditions.<sup>4</sup> We anticipate that the present novel method will be useful as a “smart” technology for selectively controlling the function of target proteins. In addition, it should prove useful in protein structure–activity studies, investigation of structural domains, and the design of novel, protein-targeting therapeutic drugs.

## Results and discussion

### Investigation of 2-phenylquinoline as a protein photo-degrading small organic molecule

In our previous work on DNA photocleavage, we found that certain 2-phenylquinoline derivatives efficiently cleaved DNA upon light activation.<sup>5</sup> Based on these findings, we hypothesized that if a 2-phenylquinoline derivative could be made to produce a radical or ROS upon photo-excitation, this compound could also be used for the degradation of protein molecules. To investigate this hypothesis, we selected 2-phenylquinoline (**1**) (Fig. 1) as the protein photo-degrading agent, and human ER- $\alpha$  as the target protein. The 2-phenylquinoline scaffold is similar to estrogen in terms of its affinity for the ER.<sup>6</sup> Modulation of ER- $\alpha$  function is an important factor in a variety of diseases, including breast cancer and osteoporosis.<sup>7</sup>

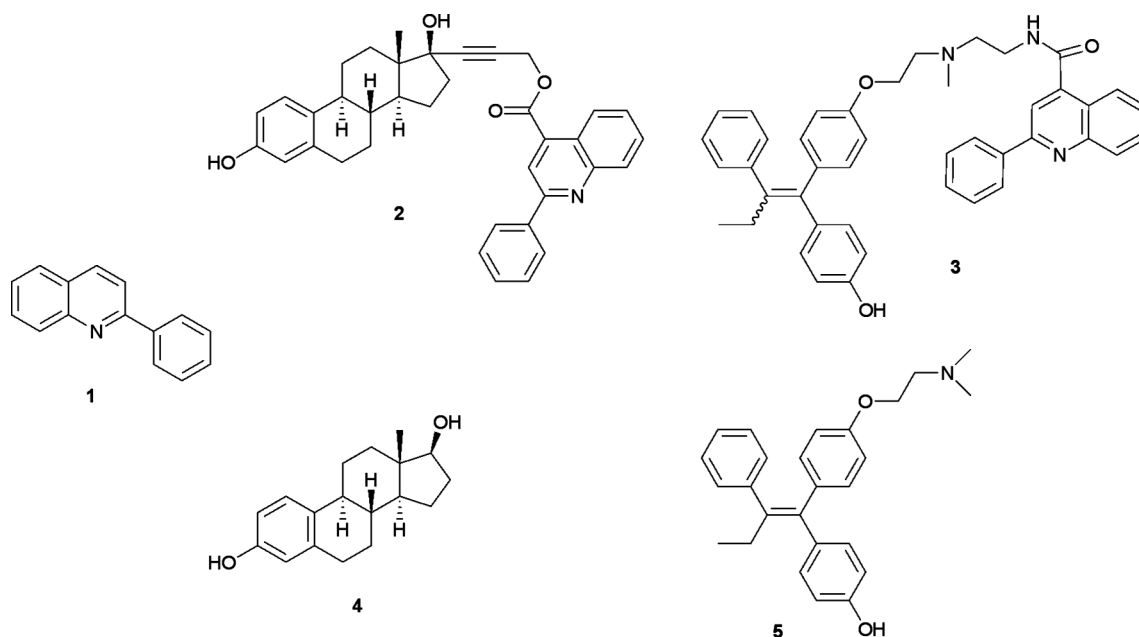
To determine if 2-phenylquinoline is capable of modulating ER- $\alpha$  function, we examined the photo-induced protein-degrading activity of 2-phenylquinoline at concentrations of 10, 3.0, 1.0 and 0.1  $\mu\text{M}$  against 1.0  $\mu\text{M}$  ER- $\alpha$  in 20% acetonitrile/Tris-HCl buffer (pH 8.0, 50 mM) using a long-wavelength UV lamp (365 nm, 100 W) for photo-irradiation. The extent of photo-degradation was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),<sup>8</sup> and the results are shown in Fig. 2. Comparison of lanes 3 and 4 with lane 2 shows that neither photo-



**Fig. 2** Photo-degradation of human estrogen receptor- $\alpha$  (ER- $\alpha$ ) by 2-phenylquinoline (**1**). ER- $\alpha$  (1.0  $\mu\text{M}$ ) was incubated with 2-phenylquinoline (**1**) in 20% acetonitrile/Tris-HCl buffer (pH 8.0, 50 mM) at 25 °C for 2 h under irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample, and analyzed by tricine-SDS-PAGE. Lane 1: size marker; lane 2: ER- $\alpha$  alone; lane 3: ER- $\alpha$  with UV; lane 4: ER- $\alpha$  + 2-phenylquinoline (**1**) (10  $\mu\text{M}$ ) without UV; lanes 5–8: ER- $\alpha$  + 2-phenylquinoline (**1**) (concentrations 10, 3, 1, and 0.3  $\mu\text{M}$ , respectively) with UV.

irradiation of ER- $\alpha$  in the absence of 2-phenylquinoline (lane 3) nor treatment of ER- $\alpha$  with 2-phenylquinoline without photo-irradiation (lane 4) resulted in a change in the SDS-PAGE profile. In contrast, lane 5 shows fading of the band corresponding to ER- $\alpha$  after exposure to 2-phenylquinoline with photo-irradiation, indicating that degradation of ER- $\alpha$  took place. These results show that 2-phenylquinoline degrades ER- $\alpha$  upon irradiation with long wavelength UV light without the need of additives, although protein degradation is incomplete.

The lack of ER- $\alpha$  degradation in the absence of light confirms that UV light functions as a trigger to initiate protein degradation by 2-phenylquinoline. In addition, it was confirmed that the degradation ability was photo-dose-dependent. The SDS-PAGE pattern obtained for 2-phenylquinoline-induced ER- $\alpha$  degradation contained faded and smeared bands, and no aggregation of the protein was observed at the top of the gels. Additionally, no



**Fig. 1** Chemical structures of 2-phenylquinoline (**1**), 2-phenylquinoline-estradiol (hybrid **2**), 2-phenylquinoline-4-hydroxytamoxifen (hybrid **3**), estradiol (**4**), and 4-hydroxytamoxifen (**5**).

peaks corresponding to degraded peptide fragments were detected by MALDI-TOF MS analysis. These results suggest that the degradation reaction occurred in a non-site-specific manner, and that ER- $\alpha$  was degraded into peptide fragments that were too small for detection by SDS-PAGE and MALDI-TOF MS analyses. Similar phenomena were reported by Jones<sup>9a</sup> and Nakanishi<sup>9b</sup> in their protein cleavages by radical species. Therefore, we conclude that 2-phenylquinoline degrades ER- $\alpha$  in a random fashion.

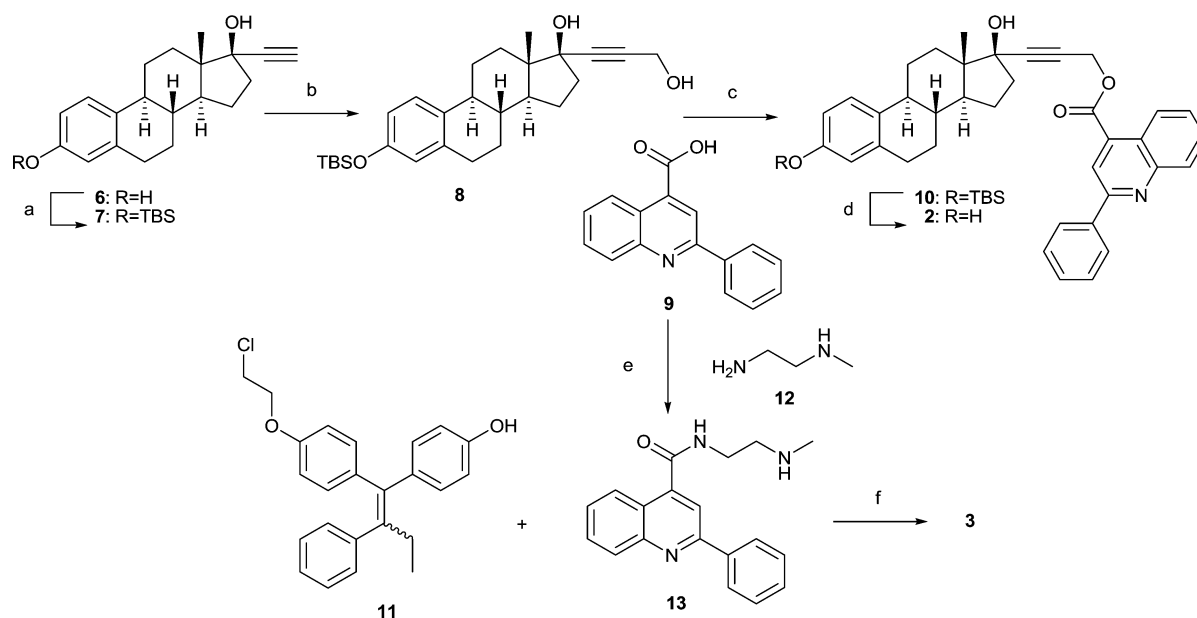
### Design and synthesis of 2-phenylquinoline-estradiol and 2-phenylquinoline-4-hydroxytamoxifen hybrids

In order to improve the protein degrading ability and selectivity of 2-phenylquinoline, we designed two hybrid molecules, consisting of 2-phenylquinoline conjugated with either estradiol (hereafter referred to as hybrid **2**) or 4-hydroxytamoxifen (hereafter referred to as hybrid **3**) (Fig. 1). Estradiol (**4**) and 4-hydroxytamoxifen (**5**) (Fig. 1) have very strong and selective affinity for ER- $\alpha$ , and function as the agonist and antagonist of ER- $\alpha$ , respectively. Hybrid molecules **2** and **3** were synthesized as shown in Scheme 1 below. Hybrid **2** was synthesized using a modified version of the procedure reported by Jones *et al.* in which an enediyne was employed as a protein cleaver.<sup>10</sup> Commercially available estradiol derivative **6** was regioselectively protected with a *t*-butyldimethylsilyl (TBS) group to produce the silylated compound **7**, which was then treated with lithium diisopropylamide (LDA), followed by addition of  $(\text{CH}_2\text{O})_n$  to furnish the primary alcohol **8**. Subsequent esterification of **8** with 2-phenylquinoline-4-carboxylic acid (**9**) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N,N*-4-dimethylaminopyridine (DMAP) produced derivative **10**. Finally, deprotection of the TBS group in **10** using tetra-*n*-butylammonium fluoride (TBAF) and AcOH yielded hybrid **2** (2-phenylquinoline-estradiol). 2-phenylquinoline-4-hydroxytamoxifen (hybrid **3**) was synthesized in a short-step

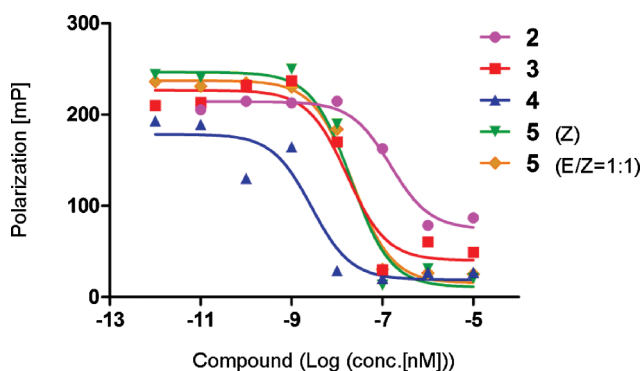
manner from the commercially available **9** and **11** of Scheme 1. **9** was amidated by the diamine derivative **12** in the presence of *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) and *N*-menthylmorpholine (NEM), yielding derivative **13**, which was then coupled with a 4-hydroxytamoxifen analog **11** using  $\text{K}_2\text{CO}_3$  and KI to produce hybrid **3** as a 1 : 1 mixture of *E* and *Z* isomers. These isomers could not be separated by practical methods such as silica-gel column chromatography.

### Selective affinity and photo-degradation capability of the hybrids against ER- $\alpha$

The binding affinity of synthetic hybrids **2** and **3** was examined using estradiol and 4-hydroxytamoxifen (*Z* isomer and a 1 : 1 mixture of *E* and *Z* isomers) as reference samples. The method employed uses fluorescence polarization to measure the capacity of a test compound to displace a high affinity fluorescent ligand, in this case fluorescent nonsteroidal estrogen, from ER- $\alpha$ . A Beacon 2000<sup>®</sup> Fluorescence Polarization Instrument with a 490 nm excitation filter and a 535 nm emission filter was utilized.<sup>11</sup> The inhibitory concentration ( $\text{IC}_{50}$ ) was determined by measuring polarization intensity as a function of test compound concentration. Results are shown in Fig. 3. The  $\text{IC}_{50}$  values were 151, 16.8, 2.8, 20.6, and 20.2 nM for hybrid **2**, hybrid **3**, estradiol, 4-hydroxytamoxifen (*Z*), and 4-hydroxytamoxifen (*E/Z* = 1/1), respectively. There was good agreement between the  $\text{IC}_{50}$  values determined for estradiol and 4-hydroxytamoxifen (*Z* isomer) and those previously reported.<sup>12</sup> These results indicated that the ER- $\alpha$  binding affinity of hybrid **2** is lower than that of estradiol, while the affinity of hybrid **3** is similar to that of 4-hydroxytamoxifen. In addition, we found that 4-hydroxytamoxifen (*Z*) and 4-hydroxytamoxifen (*E/Z* = 1/1) had quite similar affinity for ER- $\alpha$ . Furthermore, it was confirmed that the ER- $\alpha$  binding affinity of **1** was too low to be detected by the competition binding assay.



**Scheme 1** Synthesis of hybrid molecules. Reagents and conditions: (a) TBSOTf, 2,6-lutidine,  $\text{CH}_2\text{Cl}_2$ , 0 °C to rt, 2 h, 100%; (b) LDA,  $(\text{CH}_2\text{O})_n$ , THF, -78 to 0 °C, 17 h, 77%; (c) EDC, DMAP,  $\text{CH}_2\text{Cl}_2$ , 0 °C to rt, 2 h, 95%; (d) TBAF, AcOH, THF, 0 °C to rt, 1 h, 100%; (e) TBTU, NEM, DMF, rt, 20 h, 63%; (f)  $\text{K}_2\text{CO}_3$ , KI, DMF, 80 °C, 48 h, 21%.



**Fig. 3** Competition binding curves of hybrid 2, hybrid 3, estradiol (4), and 4-hydroxytamoxifen (5) against the ER- $\alpha$ /fluorescent ligand (Fluormone<sup>TM</sup> ES2) complex (ER- $\alpha$ /Fluormone<sup>TM</sup> ES2). Increasing concentrations of each competitor were incubated with 15 nM ER- $\alpha$  and 1 nM Fluormone<sup>TM</sup> ES2 for 2 h at 25 °C, followed by measurement of fluorescence polarization with a 490 nm excitation filter and a 535 nm emission filter. The IC<sub>50</sub> values were determined from non-linear regression analysis using Prism<sup>®</sup> (Graphpad Software, Inc.).

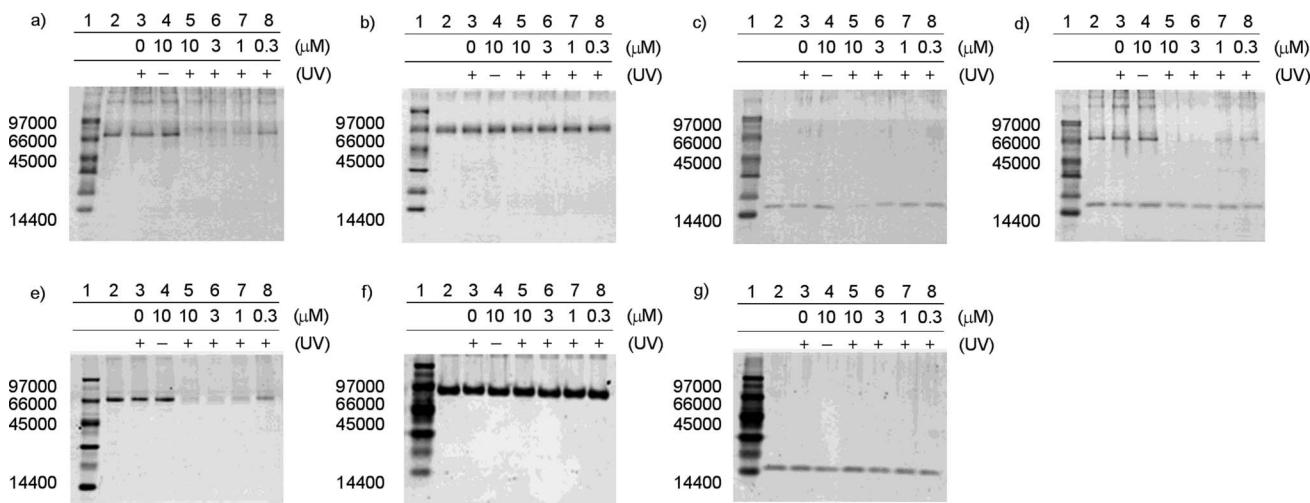
From these results, we expect that although the binding affinity of hybrid 2 was relatively low, this phenomenon might reduce undesired agonist effects of hybrid 2 toward ER- $\alpha$ , and might be beneficial for inhibition of cancer cell growth by selective degradation of ER- $\alpha$ . We also expect that the high binding affinity of hybrid 3, comparable to the antagonist 4-hydroxytamoxifen, might result in a favorable dual effect; that is, the antagonist and photo-degradation effects may act in concert to inhibit cancer cell growth. Considering the results of binding studies involving 4-hydroxytamoxifen (*Z* isomer and 1 : 1 mixture of *E* and *Z* isomers), further studies involving hybrid 3 used a 1 : 1 mixture of *E* and *Z* isomers.

Next, we evaluated 2-phenylquinoline-estradiol (hybrid 2) and 2-phenylquinoline-4-hydroxytamoxifen (hybrid 3) for target-

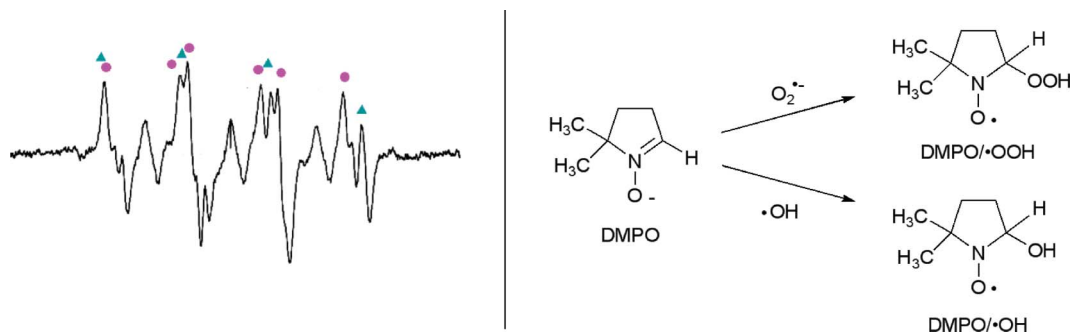
selective photo-degradation of proteins in glass vessels. Photo-induced degradation of three types of protein (ER- $\alpha$ , bovine serum albumin (BSA), and hen egg lysozyme (Lyso)) by hybrids 2 and 3 was evaluated by SDS-PAGE. The results are summarized in Fig. 4. Significant degradation of ER- $\alpha$  occurred when exposed to hybrids 2 and 3 under photo-irradiation (Fig. 4a and e). Hybrids 2 and 3 degraded ER- $\alpha$  to a much greater extent than those obtained with BSA and Lyso, which showed either no or slight degradation under photo-irradiation with hybrid 2 or 3 (Fig. 4b, c, f, and g). It was noteworthy that while Lyso was only slightly degraded by hybrid 2 (Fig. 4c) in isolation, when ER- $\alpha$  and Lyso were both present in the reaction mixture, only ER- $\alpha$  was degraded (Fig. 4d). These results clearly indicate that 2-phenylquinoline-estradiol and 2-phenylquinoline-4-hydroxytamoxifen promote selective degradation only of the target protein, ER- $\alpha$ , upon photo-irradiation, without any additives and under neutral conditions.

### Mechanistic study of selective photo-degradation

Mechanistic studies of selective photo-degradation utilized 2-phenylquinoline-estradiol hybrid 2. The activity of hybrid 2 decreased in the presence of O<sub>2</sub><sup>•-</sup> and the H<sub>2</sub>O<sub>2</sub> scavengers Tiron and KI, though these scavengers are not specific for O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>. Furthermore, photo-irradiation of hybrid 2 in the presence of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) produced products with an ESR spectrum characteristic of the DMPO-superoxide anion spin adduct DMPO/•OOH and the DMPO-hydroxyl radical spin adduct DMPO/•OH. DMPO/•OOH and DMPO/•OH are products of the reaction of DMPO with O<sub>2</sub><sup>•-</sup> and the reaction of DMPO with •OH and/or the decay of DMPO/•OOH, respectively (Fig. 5).<sup>13</sup> It was confirmed that no peaks corresponding to DMPO/•OOH and DMPO/•OH were detected by treatment of DMPO with hybrid 2 without photo-irradiation or by photo-irradiation of DMPO in the absence of



**Fig. 4** Photo-degradation of proteins by 2-phenylquinoline-estradiol (hybrid 2) and 2-phenylquinoline-4-hydroxytamoxifen (hybrid 3). Each protein (1.0  $\mu$ M) was incubated with hybrid 2 or 3 in 20% acetonitrile/Tris-HCl buffer (pH 8.0, 50 mM) at 25 °C for 2 h under irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample, and the products were analyzed by tricine-SDS-PAGE. a), b), c), d), e) f) and g) represent a) ER- $\alpha$  with hybrid 2, b) BSA with hybrid 2, c) Lyso with hybrid 2, d) ER- $\alpha$  + Lyso with hybrid 2, e) ER- $\alpha$  with hybrid 3, f) BSA with hybrid 3, and g) Lyso with hybrid 3. Lane 1: size marker; lane 2: protein alone; lane 3: protein with UV; lane 4: protein + compound (10  $\mu$ M) without UV; lanes 5–8: protein + compound (concentrations 10, 3, 1, and 0.3  $\mu$ M, respectively) with UV.



**Fig. 5** ESR spectrum obtained during photo-irradiation of hybrid **2** in the presence of DMPO. Hybrid **2** (4 mM) and DMPO (100 mM) were dissolved in 50% acetonitrile/Tris-HCl buffer (pH 8.0, 50 mM). The pink circles and the blue triangles represent the signals of DMPO•OOH and DMPO•OH, respectively.

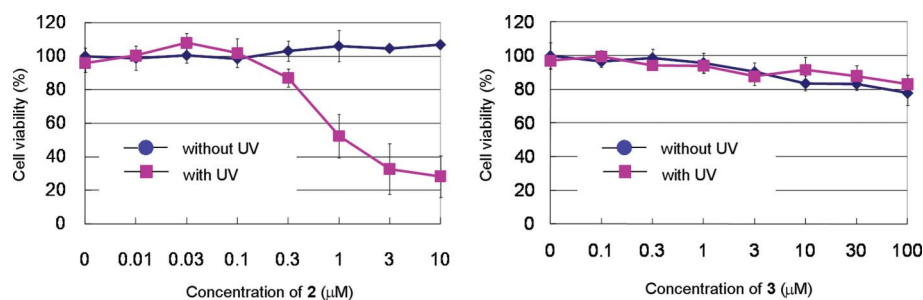
hybrid **2**. Although a singlet oxygen-mediated degradation mechanism could not be ruled out, it was found that the degradation ability of hybrid **2** in D<sub>2</sub>O (in which the lifetime of singlet oxygen is extended) was similar to that in H<sub>2</sub>O. Furthermore, similar results were obtained by using hybrid **3** in these mechanistic studies. From these results, we conclude that ER- $\alpha$  degradation by 2-phenylquinoline derivatives must be due to ROS produced by photo-excitation of 2-phenylquinoline and O<sub>2</sub>.<sup>14</sup> Importantly, the life-time of ROS are very short; therefore, target selectivity is generated by the location of the LAMTA molecules hybrids **2** and **3**.

#### Target-selective photo-degradation of ER- $\alpha$ leading to inhibition of the growth of MCF-7 breast cancer cells

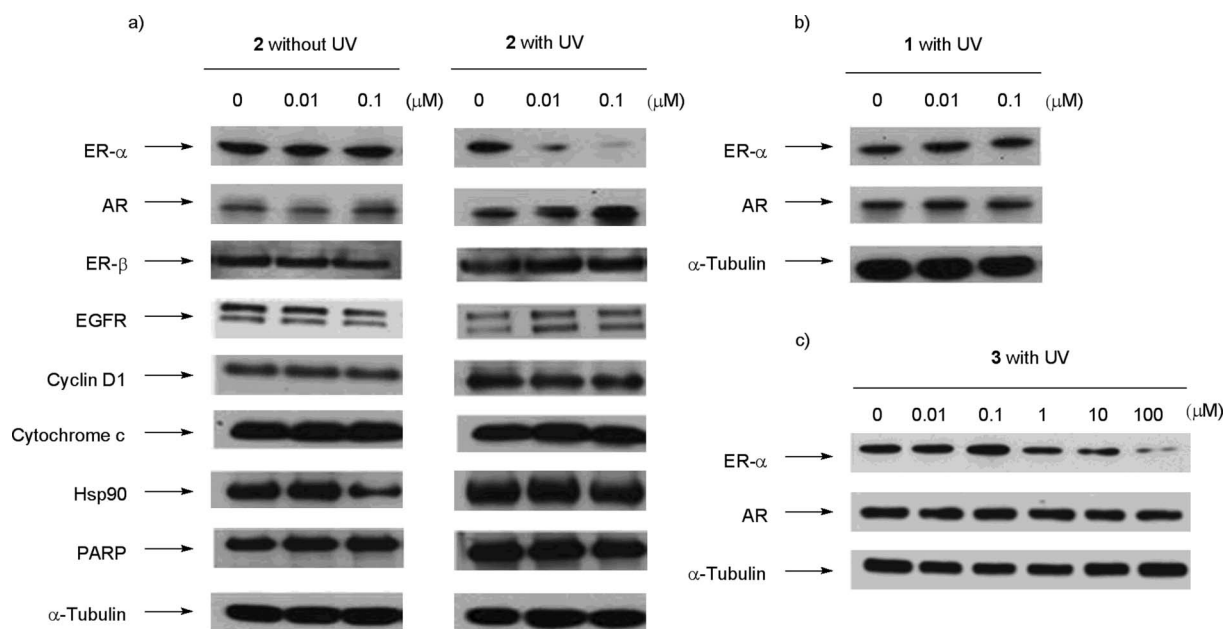
We next evaluated the activity of our 2-phenylquinoline hybrids at the cellular level. First, we utilized an MTT assay to evaluate potential cytotoxic effects of hybrids **2** and **3**. Breast cancer MCF-7 cells, which are dependent upon ER- $\alpha$  for growth, were exposed to each agent for 24 h with or without 1 h of photo-irradiation. The IC<sub>50</sub> values of hybrids **2** and **3** against MCF-7 cells without photo-irradiation were >10  $\mu$ M, and with photo-irradiation were 1.1  $\mu$ M and >10  $\mu$ M, respectively (Fig. 6). In the case of hybrid **3**, no cytotoxicity was observed at a concentration of 100  $\mu$ M with or without photo-irradiation. These results indicate that hybrids **2** and **3** are in and of themselves non-toxic, while only hybrid **2** is cytotoxic upon photo-irradiation. Based on these results, target-selective photo-degradation of ER- $\alpha$  by hybrids **2** and **3** in

MCF-7 cells was investigated by western blotting with appropriate monoclonal antibodies within the concentrations at which the hybrid compounds showed no cytotoxicity. No degradation of any of the proteins examined (ER- $\alpha$ , AR, ER- $\beta$ , EGFR, cyclin D1, cytochrome c, Hsp90, PARP, and  $\alpha$ -tubulin) was observed when hybrid **2** was not subjected to photo-irradiation (Fig. 7). On the other hand, when hybrid **2** was subjected to 1 h of photo-irradiation, only a dose-dependent degradation of ER- $\alpha$  was observed (Fig. 7a). In addition, 2-phenylquinoline (**1**) itself did not degrade ER- $\alpha$  even under photo-irradiation over a similar concentration range (Fig. 7b). These results show the importance of the hybrid structure for target-selective photo-degradation, in which 2-phenylquinoline serves as the protein degradation moiety and estradiol functions in protein recognition. Hybrid **3** exhibited a similar target-selectivity (Fig. 7c). Thus, although a higher concentration of hybrid **3** was required for the photo-degradation of ER- $\alpha$ , under photo-irradiation hybrid **3** degraded ER- $\alpha$  in a dose dependent manner but did not degrade the other proteins tested. The difference in the strength of photo-degrading activity between hybrids **2** and **3** may be a result of differences in cellular uptake.

Finally, we examined the effect of hybrids **2** and **3** on the growth of MCF-7 breast cancer cells with and without photo-irradiation under two different sets of culture conditions. Under culture condition A, MCF-7 cells were maintained for 2 days in the absence of phenol-red, a substance with a weak estrogenic activity,<sup>15</sup> and in the presence of charcoal-stripped fetal bovine serum (FBS). Under these conditions, cell growth is estradiol dependent. Cells



**Fig. 6** Effect of hybrids **2** and **3** on MCF-7 cell viability. Cells were seeded at 3000 per well in 10% FBS DMEM in a 96-well plate. After 24 h, samples were incubated with the indicated concentrations of hybrids **2** or **3** at 25 °C for 1 h with or without photo-irradiation by a UV lamp (368 nm, 15 W) placed 25 cm from the samples. Cells were then kept for 24 h at 37 °C and in 5% CO<sub>2</sub> in air, and then MTT reagent was added to each well and cells were incubated for up to 3 additional hours. The absorbance at a single wavelength of 540 nm was read on a plate reader.



**Fig. 7** Western blot analysis of the effect of 2-phenylquinoline (**1**) and hybrids **2** and **3** with or without photo-irradiation on protein levels in MCF-7 cells. Cells were treated with each compound for 1 h at 25 °C with or without photo-irradiation using a UV lamp (368 nm, 15 W) placed 25 cm from the samples. Each sample was analyzed using tricine-SDS-PAGE and immunoblotting with appropriate monoclonal antibodies.  $\alpha$ -Tubulin levels are shown as protein loading controls.

were cultured in this manner to enhance the assay sensitivity. Cells were then treated with each hybrid photo-degrading agent for 3 days. Under the second set of culture conditions (condition B), estradiol was added before treatment with the hybrid compounds. When MCF-7 cells were exposed to hybrid **2** (which contains an estradiol moiety) under culture condition A without photo-irradiation, cell growth was dose-dependent relative to hybrid **2**. These results indicate that hybrid **2** works as an agonist against ER- $\alpha$  as a result of the estradiol component of its structure. However, with 1 h of photo-irradiation under condition A, cell growth was significantly depressed (Fig. 8a). These results clearly show that photo-degradation of ER- $\alpha$  by hybrid **2** effectively induces inhibition of the growth of MCF-7 cells. Under culture condition B without photo-irradiation, MCF-7 cell growth was independent of the concentration of hybrid **2**, reaching the same cell density at all concentrations of hybrid **2** due to the presence of excess amounts of estradiol in the media. However, although inhibitory activity was not particularly high, hybrid **2** did inhibit MCF-7 cell growth with photo-irradiation due to degradation of ER- $\alpha$  (Fig. 8b). On the other hand, when MCF-7 cells were exposed to hybrid **3** (containing a 4-hydroxytamoxifen moiety) under culture condition A with or without photo-irradiation, cells did not increase in number with concentration of hybrid **3** because hybrid **3** had no agonist activity against ER- $\alpha$  (Fig. 8c). Under culture condition B without photo-irradiation, hybrid **3** inhibited MCF-7 cell growth in a dose-dependent manner. These results indicate that hybrid **3** functions as an antagonist of ER- $\alpha$  due to its 4-hydroxytamoxifen structure. Furthermore, inhibition of cell growth increased with photo-irradiation as the concentration of hybrid **3** increased (Fig. 8d). These results clearly demonstrate that hybrid **3** has dual antagonist and photo-degradation activity toward ER- $\alpha$  and thereby effectively inhibits the growth of MCF-7 breast cancer cells.

## Conclusions

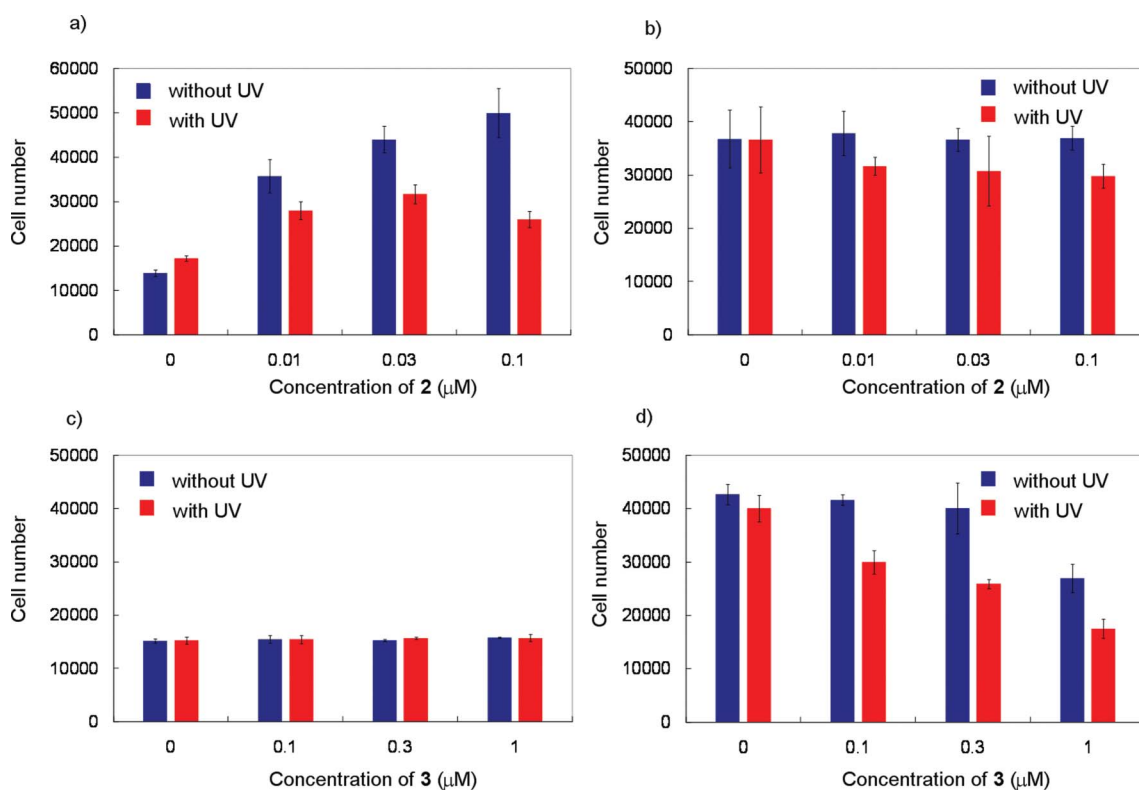
In the present studies, we developed a new method for selective degradation of a target protein, ER- $\alpha$ , by photo-irradiation using 2-phenylquinoline-estradiol and -4-hydroxytamoxifen hybrids under neutral conditions. Our results indicate that target-selective photo-degradation of proteins by LAMTA molecules may be a novel and effective way to control specific protein functions as a means of controlling cell growth. In addition, the results presented here will contribute to the molecular design of novel artificial protein photo-degradation agents, which should find wide applications in chemistry, biology, and medicine.

## Experimental section

### General procedure for chemical synthesis

17 $\alpha$ -ethynyl- $\beta$ -estradiol (**6**), 2-phenylquinoline-4-carboxylic acid (**9**), and (*E/Z*)-1-[4-chloroethoxy]phenyl]-1-(4-hydroxyphenyl)-2-phenyl-1-butene (**11**) were purchased from Sigma Co., Aldrich Co., and Santa Cruz Biotechnology, Inc. respectively. Melting points were determined on a micro hot-stage (Yanako MP-S3) and were uncorrected. Optical rotations were measured on a JASCO DIP-370 photo-electric polarimeter. <sup>1</sup>H-NMR spectra were recorded on a Varian MVX-300 (300 MHz) or a JEOL ECA-500 (500 MHz) spectrometer using trimethylsilane as internal standard unless otherwise noted. ESI-TOF Mass spectra were measured on a Waters LCT premier XE. Silica gel TLC and column chromatography were performed on Merck TLC 60F-254 (0.25 mm), Silica Gel 60 N (spherical, neutral) (Kanto Chemical Co., Inc.), and Wakosil 40C18 (Wako Pure Chemical Industries, Ltd.), respectively. Air- and/or moisture-sensitive reactions were





**Fig. 8** Effect of hybrids **2** and **3** on MCF-7 cell growth. MCF-7 cells were maintained in culture in the presence 10% charcoal-stripped FBS for 2 days, after which cells were seeded into 24-well culture plates ( $1 \times 10^4$  per well) in the same media. After 24 h, samples were incubated with the indicated concentrations of hybrids **2** or **3** with or without 1 h photo-irradiation using a UV lamp (368 nm, 15 W) placed 25 cm from the samples. a) Hybrid **2** grown under Condition A. b) Hybrid **2** grown under Condition B. c) Hybrid **3** grown under Condition A. d) Hybrid **3** grown under Condition B. For the estradiol-treated groups b) and d), estradiol (1 nM) was added 30 min before treatment with hybrid **2** or **3**. After treatment at 37 °C and in air maintained at 5% CO<sub>2</sub> for 3 days, cells were harvested and the cell number in each well was determined using a Coulter counter.

carried out under an atmosphere of argon using oven-dried glassware. In general, organic solvents were purified and dried using an appropriate procedure, and evaporation and concentration were carried out under reduced pressure below 30 °C, unless otherwise noted.

### 3-*O*-*tert*-Butyldimethylsilyl-17 $\alpha$ -ethynyl- $\beta$ -estradiol (**7**)

To a stirred solution of **6** (309 mg, 1.04 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added 2,6-lutidine (180  $\mu$ L, 1.56 mmol) and TBSOTf (290  $\mu$ L, 1.25 mmol) at 0 °C. After stirring for 2 h at 25 °C, the mixture was poured into ice-cold water. The resultant mixture was extracted with CHCl<sub>3</sub> and the extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. Purification of the residue by column chromatography (40 g of silica gel, 3/1 n-hexane/EtOAc) gave **7** (428 mg, 100%) as white solids. *R*<sub>f</sub> 0.80 (1/1 n-hexane/EtOAc); Mp. 122.5–123.5 °C;  $[\alpha]_D^{29} +5.5$  (*c* 1.22, CHCl<sub>3</sub>); <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.18 (1H, d, *J* = 8.4 Hz), 6.66 (1H, dd, *J* = 8.4 and 2.4 Hz), 6.58 (1H, d, *J* = 2.4 Hz), 2.83–2.78 (2H, m), 2.62 (1H, s), 2.38–2.12 (3H, m), 2.05–1.96 (2H, m), 1.96–1.65 (5H, m), 1.55–1.23 (4H, m), 0.96 (9H, s), 0.89 (3H, s), 0.19 (6H, s); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  153.3, 137.8, 132.9, 126.1, 119.9, 117.2, 87.5, 79.9, 74.0, 49.5, 47.1, 43.6, 39.4, 39.0, 32.8, 29.6, 27.3, 26.3, 25.7 ( $\times$  3), 22.8, 18.1, 12.7, –4.4

( $\times$  2); Anal. Calcd for C<sub>26</sub>H<sub>38</sub>O<sub>2</sub>Si: C, 76.04; H, 9.33. Found: C, 75.67; H, 9.36%.

### 3-*O*-*tert*-Butyldimethylsilyl-17 $\alpha$ -hydroxypropargyl- $\beta$ -estradiol (**8**)

To a stirred solution of **7** (435 mg, 1.06 mmol) in dry THF (11 mL) was added LDA (1.77 mL, 3.18 mmol) at –78 °C. The reaction mixture was stirred for 0.5 h at –78 °C, and then (CH<sub>2</sub>O)<sub>n</sub> (95.5 mg, 3.18 mmol) was added to the mixture. After stirring for 17 h at 25 °C, the mixture was poured into ice-cold water. The resultant mixture was extracted with EtOAc and the extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. Purification of the residue by column chromatography (40 g of silica gel, 2/1 n-hexane/EtOAc) gave **8** (359 mg, 77%) as white solids. *R*<sub>f</sub> 0.30 (1/1 n-hexane/EtOAc); Mp. 178.0–179.0 °C;  $[\alpha]_D^{29} -3.3$  (*c* 1.05, CHCl<sub>3</sub>); <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  7.11 (1H, d, *J* = 8.4 Hz), 6.61 (1H, dd, *J* = 8.4 and 2.4 Hz), 6.55 (1H, d, *J* = 2.4 Hz), 4.36 (2H, s), 2.83–2.78 (2H, m), 2.36–2.18 (3H, m), 2.06–1.97 (1H, m), 1.88–1.61 (7H, m), 1.54–1.25 (4H, m), 0.97 (9H, s), 0.87 (3H, s), 0.19 (6H, s); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  153.3, 137.8, 132.9, 126.1, 119.9, 117.2, 89.3, 84.2, 79.9, 51.1, 49.6, 47.2, 43.6, 39.4, 38.9, 33.0, 29.6, 27.3, 26.3, 25.7 ( $\times$  3), 22.9, 18.1, 12.8, –4.4 ( $\times$  2); Anal. Calcd for C<sub>27</sub>H<sub>40</sub>O<sub>3</sub>Si: C, 73.59; H, 9.15. Found: C, 73.41; H, 9.14%.

### 3-*O*-*tert*-Butyldimethylsilyl-17 $\alpha$ -hydroxypropargyl- $\beta$ -estradiolyl 2-phenylquinoline-4-carboxylate (10)

To a stirred solution of **8** (66.0 mg, 0.150 mmol) and **9** (45.0 mg, 0.181 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (1.5 mL) was added EDC (57.0 mg, 0.300 mmol) and DMAP (9.0 mg, 0.0749 mmol) at 0 °C. After stirring for 2 h, the reaction mixture was poured into ice-cold water. The resultant mixture was extracted with CHCl<sub>3</sub> and the extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. Purification of the residue by column chromatography (30 g of silica gel, 3/1 n-hexane/EtOAc) gave **10** (95.6 mg, 95%) as white solids. *R*<sub>f</sub> 0.80 (1/1 n-hexane/EtOAc); Mp. 86.0–87.0 °C; [ $\alpha$ ]<sub>D</sub><sup>27</sup> –5.1 (*c* 0.91, CHCl<sub>3</sub>); <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.75 (1H, dd, *J* = 8.4 and 1.0 Hz), 8.43 (1H, s), 8.26–8.13 (3H, m), 7.81–7.43 (5H, m), 7.04 (1H, d, *J* = 8.4 Hz), 6.60 (1H, dd, *J* = 8.4 and 2.4 Hz), 6.54 (1H, d, *J* = 2.4 Hz), 5.16 (2H, s), 2.78 (2H, m), 2.43–2.13 (3H, m), 2.11–1.91 (2H, m), 1.91–1.64 (5H, m), 1.55–1.22 (4H, m), 0.98 (9H, s), 0.89 (3H, s), 0.19 (6H, s); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  165.6, 156.7, 153.3, 149.3, 138.7, 137.7, 135.0, 132.8, 130.4, 130.0, 129.8, 128.9 ( $\times$  2), 127.9, 127.4 ( $\times$  2), 126.1, 125.2, 123.9, 120.5, 119.9, 117.2, 91.3, 80.0, 79.5, 53.7, 49.7, 47.5, 43.6, 39.4, 38.9, 33.0, 29.6, 27.3, 26.3, 25.7 ( $\times$  3), 22.9, 18.2, 12.8, –4.4 ( $\times$  2); Anal. Calcd for C<sub>43</sub>H<sub>49</sub>NO<sub>4</sub>Si: C, 76.86; H, 7.35; N, 2.08. Found: C, 76.70; H, 7.63; N, 1.94%.

### 17 $\alpha$ -Hydroxypropargyl- $\beta$ -estradiolyl 2-phenylquinoline-4-carboxylate (2)

To a stirred solution of **10** (84.0 mg, 0.125 mmol) in dry THF (1.3 mL) was added AcOH (7.0  $\mu$ L, 0.150 mmol) and TBAF (150  $\mu$ L, 0.150 mmol) at 0 °C. After stirring for 1 h, the reaction mixture was poured into ice-cold water. The resultant mixture was extracted with EtOAc and the extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. Purification of the residue by column chromatography (30 g of silica gel, 2/1 n-hexane/EtOAc) gave **2** (69.7 mg, 100%) as white solids. *R*<sub>f</sub> 0.50 (1/1 n-hexane/EtOAc); Mp. 102.0–103.0 °C; [ $\alpha$ ]<sub>D</sub><sup>27</sup> –6.5 (*c* 0.41, CHCl<sub>3</sub>); <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.75 (1H, dd, *J* = 8.4 and 1.0 Hz), 8.43 (1H, s), 8.27–8.13 (3H, m), 7.82–7.43 (5H, m), 7.07 (1H, d, *J* = 8.4 Hz), 6.61 (1H, dd, *J* = 8.4 and 2.4 Hz), 6.55 (1H, d, *J* = 2.4 Hz), 5.16 (2H, s), 4.57 (1H, s), 2.80 (2H, m), 2.44–2.12 (3H, m), 2.11–1.91 (2H, m), 1.91–1.64 (5H, m), 1.50–1.30 (4H, m), 0.89 (3H, s, Me-13); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>)  $\delta$  165.7, 156.8, 153.5, 149.1, 138.6, 138.0, 135.1, 132.1, 130.2, 130.0, 129.8, 128.9 ( $\times$  2), 127.9, 127.5 ( $\times$  2), 126.4, 125.2, 123.8, 120.6, 115.3, 112.7, 91.3, 80.0, 79.5, 53.8, 49.6, 47.5, 43.5, 39.4, 38.8, 32.9, 29.5, 27.2, 26.3, 22.9, 12.8; Anal. Calcd for C<sub>37</sub>H<sub>35</sub>NO<sub>4</sub>: C, 79.69; H, 6.33; N, 2.51. Found: C, 79.72; H, 6.54; N, 2.77%.

### *N*-(2-(Methylamino)ethyl)-2-phenylquinoline-4-carboxamide (13)

To a stirred solution of **9** (30.0 mg, 0.120 mmol) in dry DMF (1.2 mL) was added NEM (22.8  $\mu$ L, 0.180 mmol) and TBTU (46.2 mg, 0.144 mmol) at 25 °C. The reaction mixture was stirred for 1 h at 25 °C, and then a solution of **12** (31.5  $\mu$ L, 0.360 mmol) in dry DMF (1.2 mL) was added dropwise to the mixture over a period of 15 min. After stirring for 20 h, the reaction mixture was quenched with water. The resultant mixture was extracted with EtOAc and the extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. Purification of the

residue by reverse phase column chromatography (6 g of silica gel, 0/100 to 100/0 MeOH/H<sub>2</sub>O) gave **13** (23.1 mg, 63%) as white solids. *R*<sub>f</sub> 0.20 (6/1 CHCl<sub>3</sub>/MeOH); Mp. 149.0–150.0 °C; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  8.24–8.13 (4H, m), 7.93 (1H, s), 7.79–7.73 (1H, m), 7.74–7.60 (4H, m), 6.75 (1H, m), 3.66 (2H, dt, *J* = 6.1 and 5.3 Hz), 2.90 (2H, t, *J* = 6.1 Hz), 2.46 (3H, s); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  165.8, 156.9, 148.8, 143.1, 139.0, 130.2, 129.8, 129.0, 127.6, 127.3, 125.1, 123.5, 116.7, 50.4, 39.3, 36.1; HRMS (ESI-TOF) *m/z* 306.1586 (306.1606 calcd. for C<sub>19</sub>H<sub>20</sub>N<sub>3</sub>O, [M + H]<sup>+</sup>).

### (*E/Z*)-*N*-(2-((2-(4-(1-(4-Hydroxyphenyl)-2-phenylbut-1-enyl)phenoxy)ethyl)(methylamino)-ethyl)-2-phenylquinoline-4-carboxamide (3)

To a stirred solution of **11** (11.2 mg, 0.0296 mmol) in dry DMF (700  $\mu$ L) were added K<sub>2</sub>CO<sub>3</sub> (5.0 mg, 0.0362 mmol), KI (4.1 mg, 0.0247 mmol) and a solution of **13** (16.0 mg, 0.0524 mmol) in dry DMF (700  $\mu$ L) at room temperature. After stirring for 48 h at 80 °C, the reaction mixture was quenched with water. The resultant mixture was extracted with EtOAc and the extracts were washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo*. Purification of the residue by column chromatography (10 g of silica gel, 20/1 CHCl<sub>3</sub>/MeOH) gave **3** (4.1 mg, 21%, *E/Z* = 1/1) as white solids. *R*<sub>f</sub> 0.30 (20/1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  8.29–8.22 (1/2H, m), 8.21–8.15 (3/2H, m), 8.14–8.06 (2H, m), 7.96 (1/2H, s), 7.91 (1/2H, s), 7.75–7.66 (1H, m), 7.54–7.40 (4H, m), 7.17–6.99 (6H, m), 6.94–6.92 (1H, m), 6.80–6.74 (1H, m), 6.66–6.61 (1H, m), 6.60–6.54 (2H, m), 6.46–6.40 (1H, m), 6.29–6.23 (1H, m), 4.02 (1H, t, *J* = 5.5 Hz), 3.87 (1H, t, *J* = 5.5 Hz), 3.72 (1H, dt, *J* = 6.0 and 5.2 Hz), 3.67–3.64 (1H, m), 2.90–2.71 (4H, m), 2.48–2.31 (5H, m), 0.91–0.84 (3H, m); HRMS (ESI-TOF) *m/z* 648.3223 (648.3226 calcd. for C<sub>43</sub>H<sub>42</sub>N<sub>3</sub>O<sub>3</sub>, [M + H]<sup>+</sup>).

### Protein photo-degradation

Human recombinant estrogen receptor- $\alpha$  (ER- $\alpha$ ), bovine serum albumin (BSA) and hen egg lysozyme (Lyso) were purchased from Sigma Co. A UV lamp (365 nm, 100 W, Blak-ray (B-100A), UVP, Inc.) was used for the photo-irradiation. All the protein degradation experiments were performed with ER- $\alpha$ , BSA or Lyso (1.0  $\mu$ M) in a volume of 10  $\mu$ L containing 20% acetonitrile in 50 mM Tris-HCl buffer (pH 8.0) at 25 °C for 2 h under irradiation of the UV lamp placed at 10 cm from the mixture. The protein-sample levels were varied as indicated in the figure captions.

### Electrophoresis

SDS/polyacrylamide gel electrophoresis (SDS-PAGE) experiments were performed as reported.<sup>8</sup> After addition of a 4.8  $\mu$ L solution containing SDS (5%, wt/vol), glycerol (27%, vol/vol), DTT (0.5%, wt/vol) and bromophenol blue (0.007%, wt/vol) to the photoirradiated samples. Gels (8% for BSA and 12% for ER- $\alpha$  and Lyso) were run by applying 110 V for 1.5 h for BSA or 2.5 h for ER- $\alpha$  and Lyso. The gels were stained with SYPRO Ruby Protein Gel Stain (Bio-Rad Lab. Inc.) for 3 h, destained in acetic acid (7%, vol/vol) and methanol (10%, vol/vol) for 0.5 h, and then washed with water. The gels were scanned with a Molecular Imager FX (Bio-Rad Lab. Inc.) and images were processed using



Adobe Photoshop software. Molecular weight markers were used in each gel for calibration.

### ER- $\alpha$ competitor assay

Competition binding curves for the compounds **2–5** binding to ER- $\alpha$  were obtained by using ER- $\alpha$  a competitor assay kits purchased from PanVera Co. 2X Complex (15 nM ER- $\alpha$ /1 nM Fluormone<sup>TM</sup> ES2) and the indicated concentrations of each test compound were incubated in a volume of 100  $\mu$ L containing 1% dimethylsulfoxide in ES2 Screening Buffer for 2 h at 25 °C. Fluorescence polarization values were measured by the Beacon 2000<sup>®</sup> Fluorescence Polarization Instrument with a 490 nm excitation filter and 535 nm emission filter, and plotted as a function of the test compound concentration. The IC<sub>50</sub> values were obtained from non-linear regression using Prism<sup>®</sup> from Graphpad Software, Inc.

### ESR spectrometry

ESR spectrum was recorded using a Bruker BioSpin EMX EPR operating at 9.5 GHz with 100 kHz modulation. A mixture of **2** (4 mM) and DMPO (100 mM) in a volume of 1.0 mL containing 50% acetonitrile Tris-HCl buffer (pH 8.0, 50 mM) was placed in a quartz flat cell and irradiated directly inside the microwave cavity of the spectrometer using a UV lamp (365 nm, 100 W, Blak-ray (B-100A), UVP, Inc.).

### Cell culture

Human mammary cancer MCF-7 was grown at 37 °C in 5% CO<sub>2</sub> in air in DMEM medium supplemented with phenol red, L-glutamine (2 mM), penicillin (100 Units/ml), kanamycin (5 mg ml<sup>-1</sup>) and 10% fetal bovine serum.

### MTT Assay

Cells were seeded at  $3 \times 10^3$  per well in 96-well in 10% FBS DMEM. After 24 h, samples were incubated with the indicated concentration of **2** or **3** with or without of 1 h of photo-irradiation using a UV lamp (368 nm, 15 W, FL15BLB-368, Sankyo Denki Co., Ltd.) placed at 25 cm from the samples. Control cells were treated with DMSO vehicle at a concentration equal to that in drug-treated cells (final concentration,  $\leq 1\%$ ). After 24 h treated, MTT reagent was added to each well and cells were incubated for up to 3 additional hours at 37 °C. The absorbances were measured at 540 nm in a microplate reader SAFIRE (TECAN Inc.).

### Immunoblotting

$4 \times 10^5$  cells were plated on 60 mm dishes containing 10% FBS DMEM. After 24 h, samples were incubated with the indicated concentration of **1**, **2** or **3** with or without 1 h of photo-irradiation using a UV lamp (368 nm, 15 W, FL15BLB-368, Sankyo Denki Co., Ltd.) placed at 25 cm from the samples. After the incubation time, adherent cells were washed twice with ice-cold PBS buffer, scraped with a rubber policeman, collected in 1 ml PBS buffer, and centrifuged for 5 min at 3000 rpm at 4 °C. The pellet was then resuspended in 100  $\mu$ L lysis buffer (20 mM Tris-HCl (7.5 pH), 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 1mM

sodium orthovanadate, and 2 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktail (Roche Diagnostics Co.) and homogenized with ULTRA SONIC HOMOGENIZER UH-50 (STM Inc.). The lysate was centrifuged for 10 min at 14000 rpm at 4 °C. Equal amounts of protein were separated by SDS-PAGE in 10% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes Hybond<sup>TM</sup>-ECL (GE healthcare Japan Co.). Membranes were blocked with Tris-buffered saline/0.1% Tween 20 (TBST) containing 3% nonfat dry milk for 60 min at room temperature. After washing five times with TBST, membranes were incubated with appropriately diluted primary antibodies at 4 °C overnight. After washing five times with TBST, the blots were incubated with horseradish peroxidase-conjugated specific secondary antibody for 1 h at room temperature and then again washed five times. Then the complexes were visualized in Medical Film Processor FPM100 (FUJIFILM holdings Co.) using the enhanced chemiluminescence reagents Immobilon<sup>TM</sup> Western (MILLPORE Co.). The following primary antibodies were used for the detection of the specific bands: ER- $\alpha$  (D-12, Santa Cruz Biotechnology Inc.),  $\alpha$ -Tubulin (DM1A, Santa Cruz Biotechnology Inc.), AR (441, Santa Cruz Biotechnology Inc.), EGFR (1005, Santa Cruz Biotechnology Inc.), PARP (H-250, Santa Cruz Biotechnology Inc.), Hsp90 (H-114, Santa Cruz Biotechnology Inc.), ER- $\beta$  (H-150, Santa Cruz Biotechnology Inc.), Cyclin D1 (M-20, Santa Cruz Biotechnology Inc.) and Cytochrome c (556433, BD Biosciences).

### Growth inhibition assay

MCF-7 cells were maintained in phenol red free DMEM containing 10% charcoal-stripped FBS (Hyclone Inc.) for 2 days, after which cells were seeded at  $1 \times 10^4$  per well into 48-well culture plates in the same medium. 24 h later, samples were incubated with the indicated concentration of **2** or **3** with or without of 1 h of photo-irradiation using a UV lamp (368 nm, 15 W, FL15BLB-368, Sankyo Denki Co., Ltd.) placed at 25 cm from the samples. For the estradiol-treated groups, estradiol (1 nM) was added 30 min before the drug treatment. After 72 h treated, cell number in each well was counted using a Coulter Counter (Beckman Coulter Inc.).

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