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Estrogenic Analogues Synthesized by Click Chemistry

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The estrogen receptors, responsible for the effects of this hormone, are known to be able to recognize nonsteroidogenic molecules, and this has led to the development of molecules with therapeutic potential.[1] The phenomenon of nonsteroidal ligands of the estrogen receptors is also thought to play a major role in food and environmental sciences, with the wine-polyphenol resveratrol and the insecticide DDT thought to act as estrogenic substances.

It is therefore evident that it is of great interest to develop specific nonsteroidal substances that interfere with the estrogen receptors in a receptor-specific and/or tissue-specific manner and that display agonistic, antagonistic, or partial agonistic properties. Indeed, a number of strategies have been or could be employed to generate new structures, namely the screening of existing chemical libraries, the screening of natural compound libraries, novel modifications of known compounds with estrogenic potential, or the de novo generation of chemical libraries using rapid synthetic methods.

Click chemistry is an increasingly common method for rapid synthesis of novel biologically active compounds. This term, coined by Barry K. Sharpless,[2] now refers to reactions yielding the product in high yield without the need for further purification, without generating offensive byproducts, and operating in a benign solvent, usually water. In this way, it is possible to generate a plethora of new compounds reliably and thereby accelerate the process of drug discovery. Briefly, the paradigmatic "click" reaction is the [3+2] cycloaddition between an azide and an alkyne in the presence of copper (I) salts which generate the 1,4 disubstituted 1*H*-1,2,3-triazole ring in excellent yield.[3]

Three distinct observations have drawn our attention to the possibility of applying click chemistry to the synthesis of ER ligands: 1) reports that a pyrazole core can be used to build compounds that are ER ligands,[4] 2) the successful bioisosteric replacement of pyrazole with a triazole in fibronil, an insecticide acting as a GABA receptor antagonist,[5] and 3) our report that several resveratrol analogues synthesized by click chemistry retain estrogen-like activity.[6] We have therefore used the archetypical [3+2] azide-alkyne cycloaddition to link two phenol rings, bearing the hydroxyl moieties in different positions, with a distance comparable to estradiol or diethylstilbestrol.

Azides (**1**–**3**, Figure 1) were obtained by reacting commercially available amine phenols, via diazonium salt, with sodium azide. The desired ethynyl phenols (**4**–**6**, Figure 1) were obtained using Sonogashira coupling between the commercially available iodo-phenols and trimethylsilyl acetylene. Removal of the trimethylsilyl protecting group with tetrabutylammonium fluoride afforded the final alkyne derivatives.

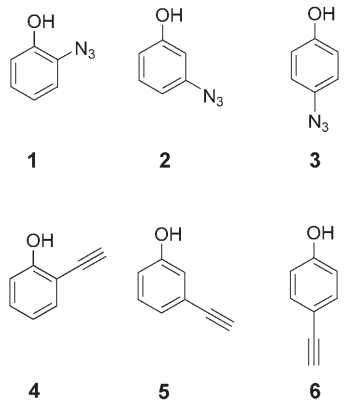

Figure 1. Azide and alkyne building blocks.

Triazole derivatives (**7**–**15**, Figure 2) were then obtained using the classical Sharpless protocol in good to moderate yields (see Supporting Information for experimental details and characterization).

To evaluate the possibility that these compounds possess estrogenic activity, we evaluated their effect on cell proliferation and viability in hormone dependent and hormone independent cell lines (MCF-7 and MDA-MB-231 respectively).[7] Cells were grown in phenol-free media in the presence or absence of increasing concentrations of compounds **7**–**15**, resveratrol, or 17β estradiol (E2). After a 5 day incubation, the MTT assay was employed to investigate the effect of the compounds on proliferation or viability (Table S1, Supporting Information). Resveratrol and compounds **7**–**15** were all highly toxic at the highest concentration tested (100 μM) on both cell lines. For resveratrol, this has been shown previously, including by our group.[6]

Most compounds were devoid of any significant activity at concentrations between 1 nM and 10 μM. On MDA-MD-231 cells, the only compounds that displayed differences were compounds **10** and **14** that appeared to be toxic. Indeed, a similar effect on these cells was observed with some click compounds similar to resveratrol in an earlier study.[6] Compound **9**

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Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

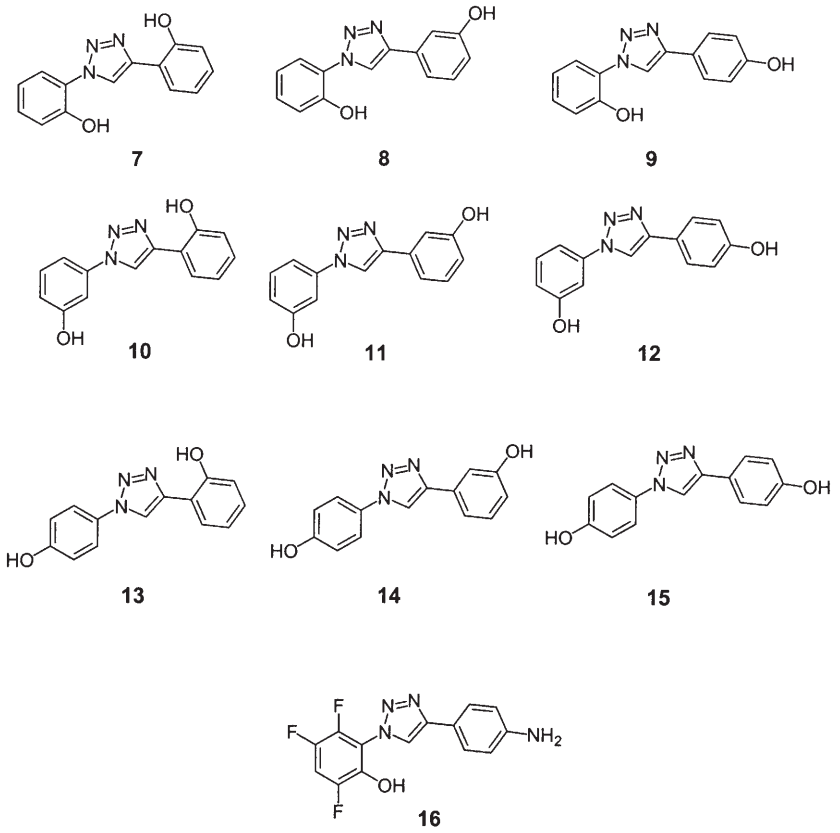

Figure 2. Triazole derivatives synthesized. Structure **16** was synthesized by different building blocks and is reported in [6].

was more toxic compared to the others in MCF-7 cells, where a concentration of 10 nM reduced viability by approximately half.

In MCF-7 but not in MDA-MD-231 cells, E2, as expected, induced an increase in proliferation compared to controls at concentrations of 10 nM and 100 nM (36±18.4% and 34±10.6% increase above control). E2 displayed an inverted bell-shape dose-response curve with maximal proliferation around 10 nM. Resveratrol also increased proliferation significantly at 100 nM (24±7% above control). Neither E2 nor resveratrol induced similar changes in MDA-MD-231 cells. Whereas most of the synthesized compounds did not display any proliferation potential in MCF-7 cells, **11** was able to increase MTT values, an index of proliferation, at concentrations between 100 pM and 1 μM (Figure 3). We have previously reported a series of click-resveratrol analogues[6] and, because of the similarity in structure, we tested the nontoxic compounds reported in that paper in the MCF-7/MDA-231 screening model. Among the compounds tested, (55 of the original 72 reported) compound **16** (Figure 2, 10 μM; labeled **Ic** in the original report)[6] was able to induce proliferation of MCF-7 (123% ±17% compared to control) and this effect was not observed in MDA-MD-231 cells (88% ±8% compared to control). All other compounds did not show any selective effect on hormone-dependent cells. Nonetheless, because of the potency and efficacy of **11**, we decided to concentrate on this compound for further characterization.

As **11** is more specific for MCF-7 cells compared to MDA-MB-231 cells, we reasoned that this might have been explained by an effect on the ER pathway. Indeed, the most accepted pharmacophore model for estrogenic activity is the presence of two hydroxyl groups, with one of them linked with an aromatic ring, separated by a distance of 10.9 Å or 12.1 Å, depending on whether a water molecule participates in binding interactions.[8] Preliminary data from molecular modeling studies (PC Spartan Package;[9] Table S2, Supporting Information) were not, however, able to shed light on this different behavior as not only **11** but also other derivatives have a comparable distance with diethylstilbestrol (**14**, **12**) or with estradiol (**13**, **9**). This might indicate an active role of the triazole ring in the binding interactions.

To confirm our biological hypothesis of the actions of **11**, we

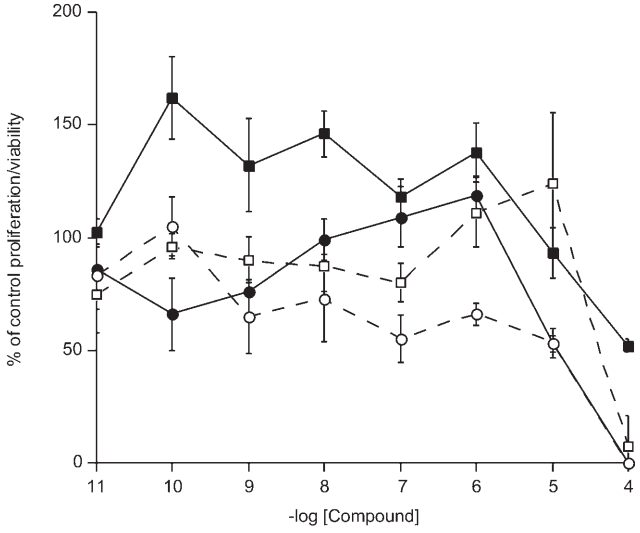

Figure 3. Concentration response curves of proliferation/toxicity of **10** and **11** evaluated by the MTT assay. See Supplementary Information for results with all compounds reported. Data are mean ±S.D. of at least 9 determinations in 3 independent experiments. ●: **10** (MCF-2); ■: **11** (MCF-7); ○: **10** (MDA-MD-231); ○: **11** (MDA-MB-231).

decided to investigate the capacity of this compound to induce ER dependent transcription using a luciferase reporter gene. As it is difficult to transfect MCF-7 cells, we employed a HeLa cell clone that constitutively expresses both ERα and ERβ

albeit at different levels (Figure 4, inset). At a concentration of 100 pM, **11** induced a significant increase in luciferase activity (Figure 4), comparable to that observed with E2 (100 nM). The

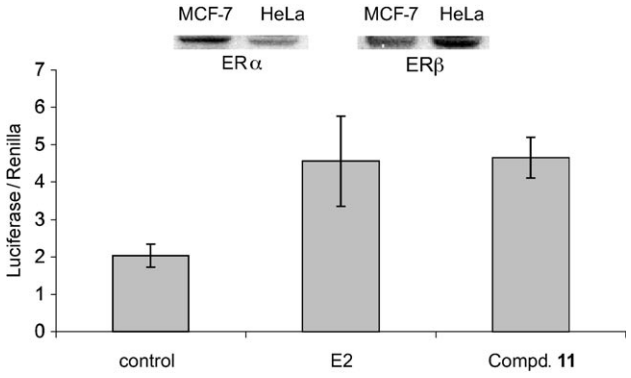

Figure 4. Transcriptional activation by E2 (100 nM) and **11** (100 pM) in HeLa cells. Activity was monitored with a ERE-luciferase reporter construct and controled with a renilla reporter construct driven by TK. Compound **10** and **13** did not elicit any significant increase of luminescence over control. Values are mean ±S.E.M. of 16–20 determinations from 3 separate experiment inset: levels of ER α or β in MDF-7 or HeLa cells as determined by Western blotting.

specificity of the assay was strengthened by the absence of any significant effect of **10** and **13** (100 pM, 111% ±36% and 130% ±18% of control, respectively) These data strongly suggest that **11** can act as a ER agonist and unmask its transcriptional activity.

Transcriptional activation is likely to depend on a direct interaction between **11** and the ER receptor, but formal proof of this assumption is required. To provide this, [^{3}H]estradiol radioligand binding assays were performed on cytosolic extracts from porcine uterus. Preliminary Scatchard analysis highlighted the presence of a high-affinity (approximate K_d 260 pM, data not shown) and a low-affinity binding site (K_d between 10 and 100 nM) for the radioligand. These two binding sites have been previously designated type I and type II, and correspond to the ER (α and β) and the bioflavonoid receptor, respectively.[10,11] In competition experiments, **11** competed selectively at the high-affinity binding site with an IC_{50} of approx. 45 pM, corresponding to a K_d of 25 pM. Unlike E2, maximal displacement was observed at 1 nM and no further displacement was observed up to 100 nM, suggesting a marked selectivity for the high-affinity binding site (Table S3, Supporting Information). As a further control, we performed competition assays with **7**, **8**, **10**, **12**, **14**, and **15** (all at 1 nM). To our surprise, these compounds, which were ineffective in our proliferation screening, all competed for 60 to 70% of binding, similar to high concentrations of estrogen (Table S3, Supporting Information). This data is in line with displacement of E2 from the low-affinity (type II) bioflavonoid binding site. Our data therefore provide strong evidence that **11** is a potent and specific ER agonist acting only on the high-affinity estrogen binding site (the estrogen receptor). On the other hand, the other compounds appear to bind to the type II receptor, although we cannot exclude that they also bind to type I.

Whereas the canonical estrogen pathway investigated above leads directly to transcriptional changes and is relatively slow, it is emerging that rapid responses can be generated by estrogens by nongenomic pathways. In detail, E2 initiates membrane signaling involving the activation of MAP kinase and PI3 kinase/Akt pathways, which then contribute to the regulation of cell proliferation and to the prevention of apoptosis.[12] Furthermore, it has been shown that E2 increases cyclin D1 expression at the transcriptional level by the PI3-kinase/Akt pathway.[12,13] Last, it has been demonstrated that interference with these ER mediated nongenomic pathways abolishes hormone-dependent breast cancer cell growth.[14]

To investigate the activation of the MAP kinase and the PI3 kinase/Akt pathway, we examined the phosphorylation of Erk and Akt in MCF-7 cells upon stimulation with **11** (100 pM and 1 nM) and with E2 (100 nM). Indeed, both **11** and E2 were able to activate this pathway within 5 min, and, as expected, 100 pM **11** was the most potent in inducing the phosphorylation of Erk (densitometric quantification with NIH Image for Erk: E2 151% ±15% and **11** at 100 pM 160% ±20% of control values; $n=3$). Similarly, within 5 min, treatment of MCF-7 with either compound induced a slight activation (that is, phosphorylation) of Akt (densitometric quantification for Akt: E2 149% ±4% and **11** at 100 pM 125% ±5% of control values; $n=3$). Last, we decided to investigate whether cyclin D1 was affected by treatment with **11** in MCF-7 cells upon longer incubations (6 h), as reported for E2. (Figure 5) Indeed, all treatments induced an increase in cyclin D1 expression, with 100 pM **11** being the most efficacious (densitometric quantification for cyclin D1: E2 119% ±13% and **11** at 100 pM 148% ±21%; $n=3$). As a specificity control, all experiments were also performed with **10**, which, at a concentration of 100 pM, was unable to elicit any effect on Erk phosphorylation, Akt phosphorylation, or cyclin D1 expression, Therefore, it would be interesting to speculate that the effects induced by **11** on proliferation are a result of the activation of both genomic and nongenomic pathways.

In conclusion, we have shown that click chemistry is applicable to estrogen receptor ligands, as demonstrated by the picomolar potency of one of the compounds synthesized (**11**). The lead compound reported here (**11**) does not appear to display specificity in terms of genomic or nongenomic effects. This would be expected as neither resveratrol or diethylstilbestrol is specific. Compound **11**, however, is specific for the ER, as it does not bind to the type II bioflavonoid receptor. Although we have fully characterized **11**, we cannot rule out that one of the other synthesized compounds also possess estrogenic or antiestrogenic activity. Indeed, our initial screening (proliferation of MCF-7 or MDA-MD-231 cells) might mask an estrogenic effect in cases where the drug affects both proliferation and cell death pathways. Yet, the importance of our observation is that the introduction of a triazole ring is compatible with binding to estrogen receptors. It will therefore be of great interest

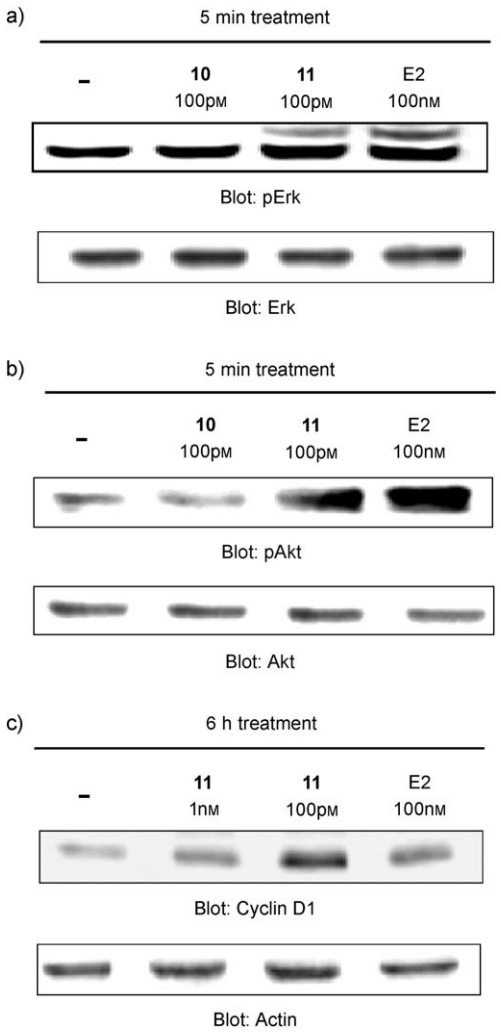

Figure 5. Activation of nongenomic pathways by E2 or **11** in MCF-7 cells. Western blots are representative of at least 3 separate experiments that yielded similar results. Experiments were performed as outlined in the Supplementary Information section.

to apply this rapid synthetic procedure to generate analogues of compounds that discriminate between receptor subtypes or that discriminate between genomic and nongenomic effects.

Acknowledgements

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Keywords: click chemistry · diethylstilbestrol · estradiol · estrogen receptor · triazole

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