# 4 Pharmacological Evaluation

The novel compounds synthesized in this work were tested for their pharmacological effects, to be precise, agonistic and antagonistic effects in MCF-7-2a cell line by a luciferase assay system.

## 4.1 Luciferase Assay System

### 4.1.1 MCF-7-2a cell line

In the early1970s, the human breast cancer cell line MCF-7 was derived from a pleural effusion of a patient with metastatic breast cancer and was shown to contain the ER [Soule, 151; Brooks, 152]. Since then it has become a prominent model system of estrogen positive breast cancer. Because of the high hormone receptor content of 70 - 90 fmol/mg protein, it is called a hormone-dependent cell line [Horwitz, 153 and 154]. The first ER cDNA was cloned from these cells [Walter, 155; Green, 16] and they yielded an estrogen-induced gene product, pS2, which was characteristic for human breast cancer cells [Masiakowski, 156]. Later, this ER was classified as ERα when a second ERβ was identified in 1996 [Kuiper, 18; Mosselman, 19]. MCF-7 cells appear to contain only the ERα subtype [Watanabe, 157]. The usefulness of the MCF-7 cell line as an investigative tool led to its adoption in laboratories worldwide. Several decades of use in independent laboratories have facilitated the evolution of distinct MCF-7 lineages [Gooch, 158; Burow, 159]. The MCF-7 cell line is the most commonly used breast cancer cell line in the world [Burdall, 160].

The MCF-7-2a cell line was obtained from ER-positive MCF-7 cells stably transfected with the plasmid $ERE_{wtc}$luc (Meyer, 161 and 162; Koop, 163). The plasmid contains the estrogen response element (ERE) of the DNA as the enhancer sequence and a reporter sequence, which codes for luciferase.

It is worthy to note that an other cell line with a similar name MCF-7-2A was identified and characterized as an estrogen independent subclone of the MCF-7 cell line by long-term growth in estrogen-free media (Pink, 164). MCF-7-2A cells express high levels of estrogen receptor (477 fmol/mg protein), not only the wild-type 66 kDa ER but also an unique 80 kDa ER containing a duplication of exons 6 and 7 in the LBD, and which can be reduced by growth in 10 nM 17 β-estradiol (201 fmol/mg protein) (Pink, 164 and 165). With its unique characteristic that the MCF-7-2A cells constitutively activate an ER-responsive luciferase reporter construct in the absence of any estrogens and this activation can be blocked by either 4-hydroxytamoxifen or ICI 164,384,

the MCF-7-2A cells also provide a unique model to use in the study of ER action and the development of estrogen-independent growth in human breast cancer cells (Pink, 164).

### 4.1.2 Luciferase assay

Luciferase is one of the usually used gene reporters. Reporter genes are applied commonly in cell biology to study gene expression and regulation. Luciferase genes have been cloned from bacteria, beetles (including firefly), Renilla, Aequorea, Vargula and Gonyaulax (a dinoflagellate). Of these, only the luciferases from bacteria, firefly and *Renilla* have found general use as indicators of gene expression. Among them, firefly luciferase is the most widely used bioluminescent reporter due to that its enzyme activity is closely coupled to protein synthesis, and the luminescence assay is rapid, sensitive and convenient [Wood, 166]. In the firefly luciferase assay, this monomeric enzyme catalyzes a two-step chemiluminescent reaction to yield light (Scheme 44). The first step is activation of the luciferin (luciferyl carboxylate) by ATP to yield a reactive mixed anhydride. In the second step, this activated intermediate reacts with oxygen to create a transient dioxetane that breaks down to the oxidized products, oxyluciferin and $CO_2$, with producing a flash of light [de Wet, 167]:

$$\text{Luciferase} + \text{Luciferin} + \text{ATP} + Mg^{2+} \rightarrow \text{Luciferase•Luciferyl-AMP} + PP_i$$
$$\text{Luciferase•Luciferyl-AMP} + O_2 \rightarrow \text{Luciferase} + \text{Oxyluciferin} + \text{AMP} + CO_2 + \mathbf{h\nu}$$

**Scheme 44**

This initial burst of light decays over about 15 seconds to a low level of sustained luminescence, which can be increased by coenzyme A to yield maximal luminescence intensity that is nearly constant for at least 1 minute [Promega Co., 168] and can be quantified with a luminometer. An optimized assay containing coenzyme A generates steady-state luminescence in less than 0.3 seconds with linearity to enzyme concentration over a 100 million-fold range. The assay sensitivity allows quantitation to less than $10^{-20}$ moles of enzyme [Wood, 166].

In the present work the luciferase gene from the firefly *Photinus pyralis* was used. It is contained in the plasmid $ERE_{wtc}$luc which was cloned by Mayer [Mayer, 161]. With this plasmid $ERE_{wtc}$luc, the ER-positive MCF-7 cell line was stably transfected to become the MCF-7-2a cell line. In a luciferase assay with MCF-7-2a cells, an active substance binds to the LBD of the ER and induces a conformational change in the ER. Then the receptors

dimerize as homodimers (ERα/ERα) and this dimerized ER binds to the EREs present in the plasmid. This leads to the expression of luciferase, which well correlates with the estrogenic potency of the active substance [Hafner, 169; von Angerer, 170]. The maximum of the luciferase expression is reached with the stably transfected cells after the 42-nd hour of culture. Therefore, the cells are destroyed by a lysis buffer after incubation with the test substance for 50 hours. The lysate is centrifuged to remove the bigger cell fragments and the supernatant is measured for luciferase activity. The measurement of the available mass of luciferase in the supernatant is performed by addition of the luciferase assay reagent, by which a two-step chemiluminescent reaction catalysed by luciferase as described above is triggered. The produced light is measured with a luminometer with automatic substrate injection. The intensity of the generated light decays rapidly after the enzyme and substrates are combined and is greatly increased by coenzym A contained in the substrate. The wavelength of the emitted light range from 490 to 630 nm. The measurement of the relative light output (unity: relative light units: RLU) is performed for a period of 10 seconds in the whole sensitive range of the Luminometer (390 - 520 nm).

## 4.2 Evaluation of Agonistic and Antagonistic Effects

### 4.2.1 Agonistic effect

Luciferase assays, one form of reporter gene assays, are based on the ability of a compound to stimulate ER-dependent transcriptional activity. Thus, the expression of luciferase is a result of the molecular cascade of events implicated in receptor activation, and as such provides a more integral indication of the estrogenic activity of a compound. Therefore, the evaluation of the agonistic effect of a substance is performed usually by determining whether and how much the stimulation of luciferase expression is induced by the investigated substance depending on the concentration of the substance. For this purpose the MCF-7-2a cell line is incubated with different concentrations of the test substance and as well with the pure solvent as background and $10^{-8}$ M of E2 as a control. The assay ends by determining the luciferase activity (RLU), and the RLU-values are correlated with the quantity of protein (quantified according to Bradford [Bradford, 171]) of each sample with the mass of luciferase. Estrogenic activity is expressed as percent activation of a $10^{-8}$ M E2 control (100%) and based on pure solvent background (0%). The activation of the luciferase

expression induced by E2 is dependent on the concentration and reaches its maximum at a concentration of $10^{-8}$ M as depicted in figure 30.

An agonist will induce increased activation of the luciferase expression within a certain range of concentrations. The concentrations of the test compounds range from $10^{-10}$ to $10^{-5}$ M. In order to compare the potential agonistic effect of different compounds, usually two values are used, namely, the relative activation value at the concentration of $10^{-6}$ M and the $EC_{50}$ value, the compound concentration causing 50% relative activation.

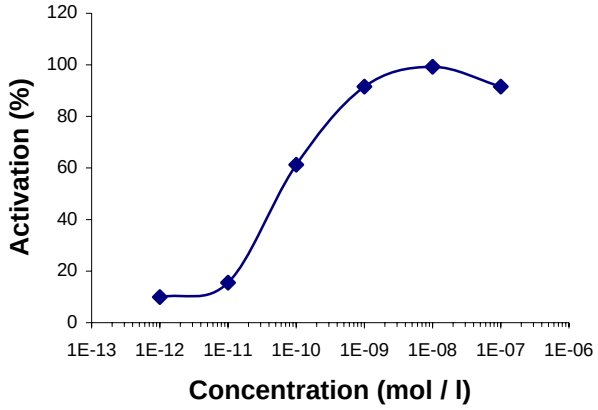



**Figure 30**. Relationship between the activation of the luciferase expression induced by E2 in the MCF-7-2a cell line and the concentration of E2.

### 4.2.2 Antagonistic effect

The antagonistic effect of an active substance indicates the competitive ability to inhibit the binding of E2 to the ER and so to reduce the stimulation of E2. Embodied in a luciferase assay, this effect is reflected by the reduced activation of the luciferase expression induced by E2 in the presence of the active substance. In order to determine the potential antagonistic effect of the compounds with a luciferase assay, the same test model as for the determination of the agonistic effect is employed. Herein the MCF-7-2a cells are incubated with different concentrations of the test substance and with a fixed concentration $10^{-9}$ M of E2. After complete assay, the obtained relationship between the activation of the luciferase expression and the concentration of test substance shows the potential antagonistic effect of the test substance. An antagonist will inhibit the activation of the luciferase expression induced by E2 in a concentration-dependent manner (see Figure 31).

The concentrations of the tested compounds range from $10^{-10}$ to $10^{-5}$ M. The relative activation value at the concentration of $10^{-6}$ M and the $IC_{50}$ value, the compound concentration causing 50% inhibition of the gene activation induced by $10^{-9}$ M E2, are also drawn to compare the potential antagonistic effect of different substances.

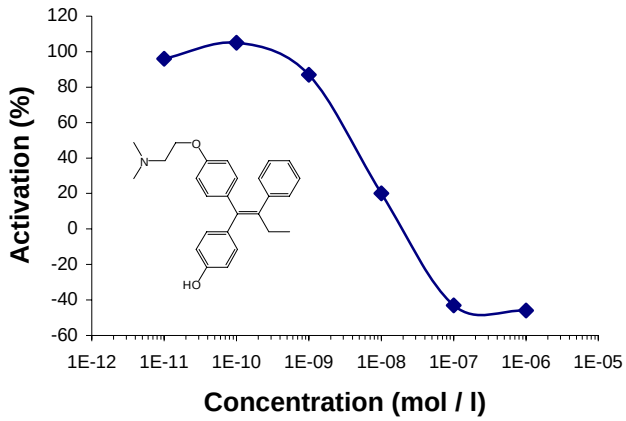


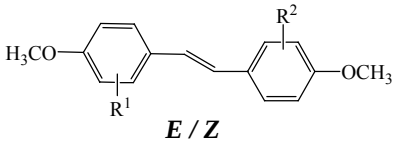

**Figure 31**. Antagonistic effect of 4-OH-tamoxifen in the MCF-7-2a cell line [Lubczyk, 84]

## 4.3 Results, Discussion and Conclusion

All target compounds were tested for their estrogenic and antiestrogenic effects in MCF-7-2a cells. These biological activities of tested substances were evaluated by the corresponding $EC_{50}$ / $IC_{50}$ values and the relative gene activation at the substance concentration of $10^{-6}$ M (1 µM).

### 4.3.1 Halo-substituted 4,4'-dimethoxystilbenes

Halo-substituted 4,4'-dimethoxystilbenes are precursors for producing the target dihydroxylated stilbenes. In order to comprehensively investigate and compare the influence of halo-substituents and methoxy as well as hydroxy on estrogenic / antiestrogenic activities of the stilbene derivatives in MCF-7-2a cells, several of 4,4'-dimethoxystilbenes were evaluated. Their $EC_{50}$ / $IC_{50}$ values and relative activations at the concentration of 1 µM are collected in table 12:




***E / Z***

**Table 12**. Evaluation for Agonistic and Antagonistic Effects of Halo-substituted Dimethoxy-stilbenes by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1μM

| Compound | $R^1$ | $R^2$ | Form | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|---|---|
| | | | (*E / Z*) | $EC_{50}$ (nM) | 1μM (%) | $IC_{50}$ (nM) | 1μM (%) |
| **9** | 2-F | 2-F | *E* | 2620 | 29.4 | - | 128.1 |
| **10** | 2-Cl | 2-Cl | *E* | 447 | 66.6 | - | 103.9 |
| **11** | 2,6-$Cl_2$ | 2,6-$Cl_2$ | *E* | 375 | 61.2 | - | 92.0 |
| **23** | H | 2-F | *E* | 7230 | 15.8 | - | 105.3 |
| **23** | H | 2-F | *Z* | 9580 | -1.7 | - | 108.1 |
| **24** | H | 2-Cl | *E* | 935 | 51.9 | - | 178.0 |
| **24** | H | 2-Cl | *Z* | 1410 | 27.3 | - | 96.6 |
| **26** | 2-F | 2,6-$Cl_2$ | *E* | 7350 | 22.9 | - | 120.0 |
| **27** | 2-Cl | 2,6-$Cl_2$ | *E* | 421 | 58.7 | - | 91.6 |

These stilbenes possess significant estrogenic effects. Each of them induced a relative gene activation of more than 50% in a concentration range of $10^{-10}$-$10^{-5}$ M compared with E2 (100%). Therefore, each substance exhibited a corresponding $EC_{50}$ value, by which the influence of halo-substituents on estrogenic activities can be clearly ranked (Figure 32):

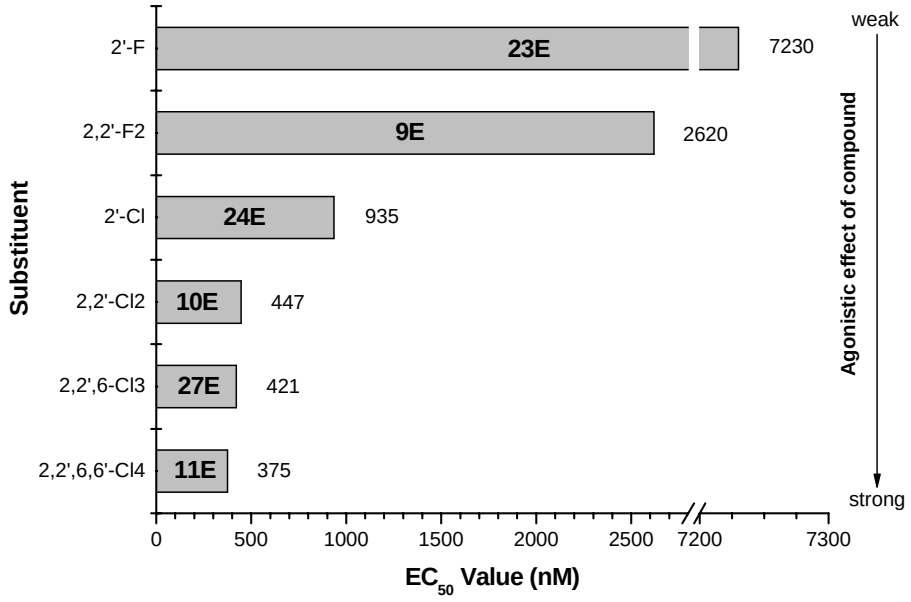


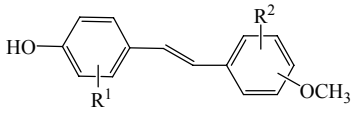

**Figure 32**: Influence of halo-substituents on estrogenic effects evaluated by $EC_{50}$ values.

4,4'-Dimethoxystilbenes with fluoro-substituents showed weaker agonistic effects, while those with chloro-substituents manifested stronger agonistic effects. More chloro-substituents signified higher transcriptional activities. But the relative activations induced by these dimethoxystilbenes at the concentration of 1 µM were lower than 67%. One of the reasons for these lower activities is that no necessary "anchor" group, e. g. phenolic hydroxy group, exists in their structures. This will be demonstrated by following test results (see Section 4.3.2, 4.3.3 and 4.3.4). In addition, the *E*-isomer was estrogenically more active than the *Z*-isomer.

None of these dimethoxystilbenes showed an antagonist activity in MCF-7-2a cells.

### 4.3.2 Halo-substituted 4-hydroxy-4' or 6'-methoxystilbenes

Compounds **33** - **40** are novel substituted stilbenes bearing a hydroxy group in 4-position of one phenyl ring and a methoxy group in the other phenyl ring and containing at least one fluoro or chloro substituent in 2 or 6 (2' or 6')-position of each stilbene. Also each of them tested for estrogenic effect in the MCF-7-2a cell line exhibited a corresponding $EC_{50}$ value and showed different estrogenic effect, which was also indicated by the different gene activation at the concentration of 1µM (see Table 13).




**33 - 40 (*E*)**

**Table 13**. Evaluation for Agonistic and Antagonistic Effects of Compounds **33 - 40 (*E*)** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1μM

| Compound | $R^1$ | $R^2$ | $OCH_3$ | Agonistic effect | | Antagonistic Effect | |
|---|---|---|---|---|---|---|---|
| | | | | $EC_{50}$ (nM) | 1μM (%) | $IC_{50}$ (nM) | 1μM (%) |
| **33** | H | 2-F | 4- | 702 | 58.3 | - | 187.9 |
| **34** | H | 2-Cl | 4- | 155 | 162.4 | - | 150.6 |
| **35** | H | 2,6-$Cl_2$ | 4- | 301 | 95.6 | - | 79.0 |
| **36** | H | 2,4-$Cl_2$ | 6- | 3080 | 33.3 | - | 93.1 |
| **37** | 2-Cl | 2,4-$Cl_2$ | 6- | 4960 | 8.9 | - | 90.6 |
| **38** | 2,6-$Cl_2$ | H | 4- | 401 | 83.6 | - | 113.2 |
| **39** | 2,6-$Cl_2$ | 2-F | 4- | 222 | 97.6 | - | 118.9 |
| **40** | 2,6-$Cl_2$ | 2-Cl | 4- | 139 | 92.1 | - | 118.0 |

Among these stilbenes, **34** bearing a chloro in 2'-position of the 4'-methoxyphenyl side is the strongest agonist with an $EC_{50}$ value of 155 nM and a relative activation of 162% at the concentration of 1μM. Introducing a second chloro into this structure in 6'-position to produce **35** lowered the estrogenic effect. The replacement of the chloro in **34** by a fluoro to produce **33** also resulted in lower agonist activity. For the 2'-fluorostilbene derivatives, more 2 or 6-chloro substituents increased the estrogenic effects with reducing the $EC_{50}$ values, e. g. $EC_{50}$ for **39** (222 nM) < $EC_{50}$ for **33** (702 nM); For the 2'-chlorostilbene derivatives, this further substitution strategy was not very successful, e. g. the $EC_{50}$ of **40** (139 nM) was similar to that of **34** (155 nM). A further comparison of the $EC_{50}$ values for **38** (402 nM) > **39** (222 nM) > **40** (139 nM) indicates that both chloro and fluoro are effective substituents for estrogenically active stilbenes, but the chloro is more effective than the fluoro (see Figure 33).

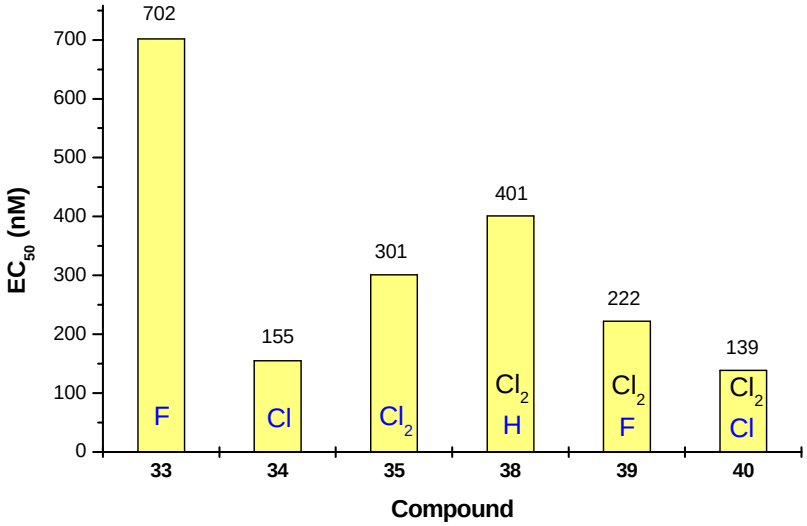



**Figure 33**. Influence of substituents on $EC_{50}$ for compounds **33** - **35** and **38** - **40**

In particular, the localization of the methoxy group in these stilbene derivatives is critical for the potential estrogenic effect. Stilbenes bearing a 6'-methoxy group e. g. **36** and **37**, showed much lower estrogenic effect than other stilbenes bearing a 4'-methoxy group, even though they comprise the same effective chloro substituents.

The two stronger agonists **34** and **40** induced the maximal activation of luciferase expression at the concentration of 2 µM and 1 µM, e. g. **34** depicted in figure 34. The other stilbenes exhibited the increasing activations from the concentration of 0.01 to 10 µM as **33** depicted in figure 34.

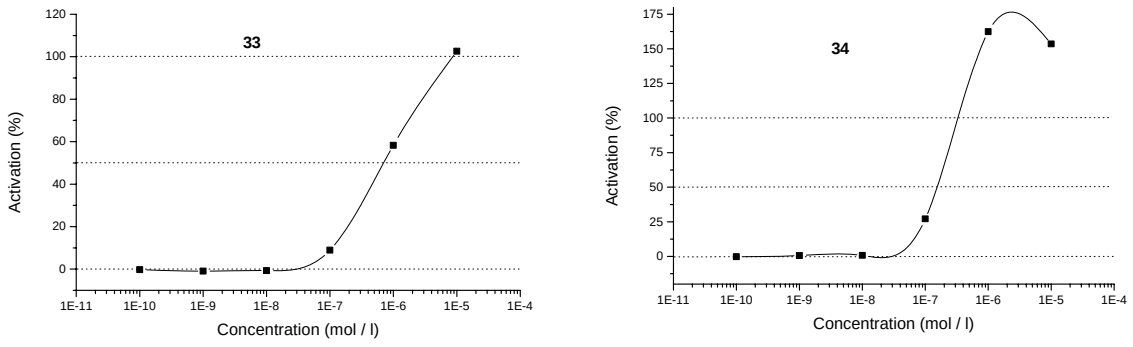


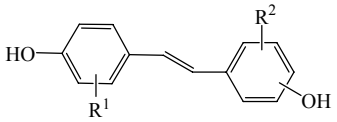

**Figure 34**. Agonistic effects of **33** and **34** tested in the concentration range of $10^{-10}$-$10^{-5}$ M.

None of the compounds **33** - **40** was antagonistically active in MCF-7-2a cells. In contrast, stilbenes **33** and **34** showed at the concentration of 1 µM even a "superagonist" activity, *i. e.*

higher activity than 1 nM E2 used in these tests, as was the case for resveratrol [Basly, 172; Gehm, 173].

### 4.3.3 Substituted 4,4'(3')-dihydroxystilbenes

Like compounds **33** - **40**, the dihydroxystilbenes **41** - **51** were agonistically active in MCF-7-2a cells. They exhibited an agonistic effect with $EC_{50}$ in the range of 40 - 1190 nM except compound **48** that induced a range of activation under 50%. The relative gene activation at the concentration of 1 µM induced by **41** - **51** ranged from 35% up to 212% (see Table 14).




**41 - 51 (*E*)**

**Table 14**. Evaluation for Agonistic and Antagonistic Effects of Compounds **41 - 51 (*E*)** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1µM

| Compound | $R^1$ | $R^2$ | OH | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|---|---|
| | | | | $EC_{50}$ (nM) | 1µM (%) | $IC_{50}$ (nM) | 1µM (%) |
| **41** | H | H | 4- | 249 | 118.9 | - | 96.3 |
| **42** | H | 2-F | 4- | 1190 | 43.9 | - | 91.8 |
| **43** | H | 2-Cl | 4- | 40 | 130.8 | - | 152.9 |
| **44** | 2-F | 2-F | 4- | 79 | 212.5 | - | 90.4 |
| **45** | 2-F | 2-Cl | 4- | 444 | 83.6 | - | 106.2 |
| **46** | 2-Cl | 2-Cl | 4- | 117 | 96.5 | - | 86.0 |
| **47** | 2-F | 2,6-$Cl_2$ | 4- | 177 | 74.8 | - | 94.8 |
| **48** | 2-Cl | 2,6-$Cl_2$ | 4- | - | 35.5 | - | 99.7 |
| **49** | 2,6-$Cl_2$ | 2,6-$Cl_2$ | 4- | 94 | 82.3 | - | 96.3 |
| **50** | 2-F | H | 3- | 246 | 155.8 | - | 138.9 |
| **51** | 2-Cl | H | 3- | 1030 | 49.2 | - | 108.8 |

The chloro or fluoro substituents enhanced the estrogenic effect only in some cases. Six more active agonists of the 4,4'-dihydroxystilbenes **41** - **49** are graphed in figure 35 in the

order of reducing $EC_{50}$ values. Among them, **41** without halo-substituent is the weakest agonist with an $EC_{50}$ value of 249 nM, while **43** with one chloro is the strongest agonist with an $EC_{50}$ value of 40 nM. The symmetric substituted 4,4'-dihydroxystilbens **44** (2,2'-$F_2$), **49** (2,2',6,6'-$Cl_4$) and **46** (2,2'-$Cl_2$) are stronger agonists than the asymmetric **47** and non-substituted **41**. Though the asymmetric 4,4'-dihydroxystilbens **42**, **45**, **48** bear one or more substituents, they showed lower estrogenic effect than non-substituted **41**.

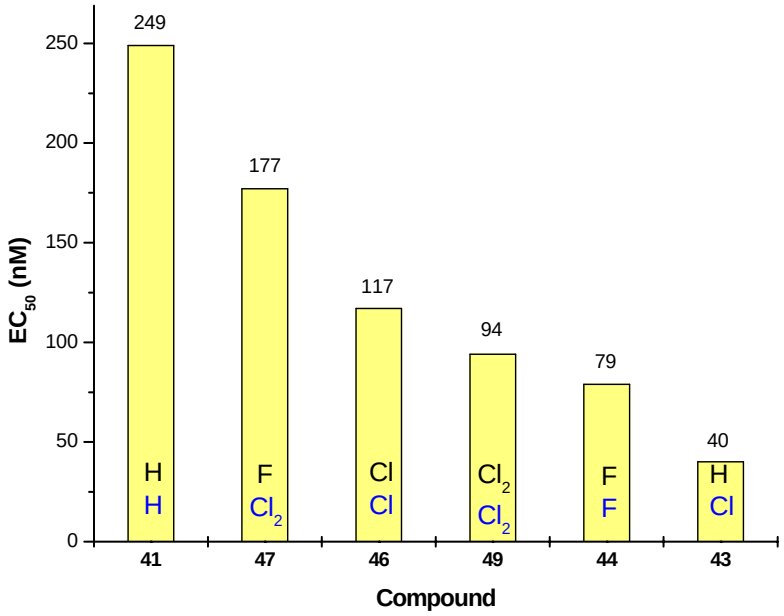



**Figure 35**. Influence of substituents on $EC_{50}$ values for compounds **41, 43, 44, 46, 47** and **49**

The influence of the OH-position in the phenyl ring on estrogenic effects depended on the substituents. The fluoro-substituted **42** bearing a 4-OH showed a lower estrogenic effect than **50** bearing a 3-OH, while the chloro-substituted **43** bearing a 4-OH established a much higher estrogenic effect than **51** bearing a 3-OH. Considering all substituents in this type of stilbenes, the symmetric stilbenes appeared to be estrogenically more active than the asymmetric stilbenes.

Some active stilbenes already induced more than 100% of the relative gene activation at the concentration of 1 µM. Such an activation was induced by the others also at the concentration of 10 µM (see Figure 36 and 37).

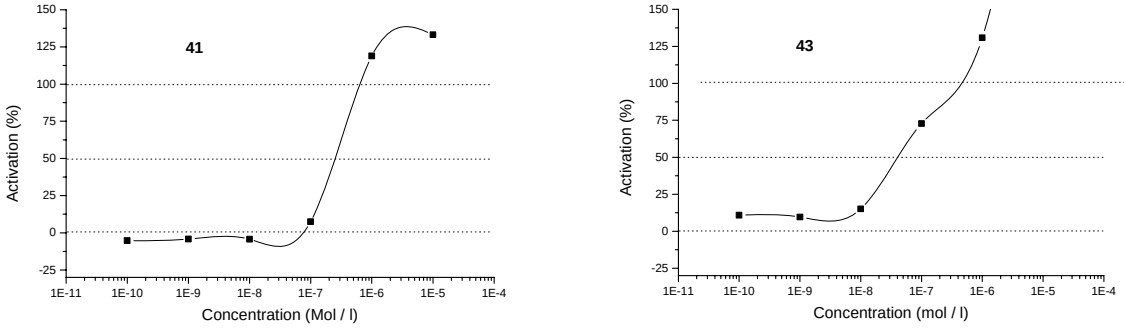



**Figure 36**. Estrogenic effect of the compounds **41** and **43** in MCF-7-2a cells.

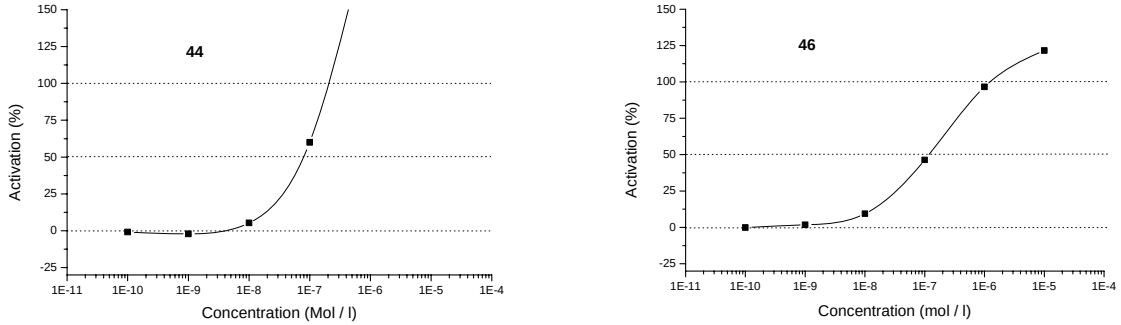



**Figure 37**. Estrogenic effect of the compounds **44** and **46** in MCF-7-2a cells.

None of the tested dihydroxystilbenes established significant antagonist activity in MCF-7-2a cells. The best agonist **43** showed also a "superagonist" activity in the presence of E2 with a gene activation of 152% at the concentration of 1 μM.

### 4.3.4 Comparison of estrogenic activities of halo-substituted dimethoxystilbenes and hydroxylated stilbenes

Halo-substituted methoxy or hydroxystilbenes showed significant but different agonist activities in the MCF-7-2a cell line. The estrogenic activities of stilbene derivatives have obvious relevance to the phenol component. This is embodied in a comparison of $EC_{50}$ values of *E*-4,4'-dimethoxystilbenes and *E*-4,4'-dihydroxystilbenes (see Figure 38).

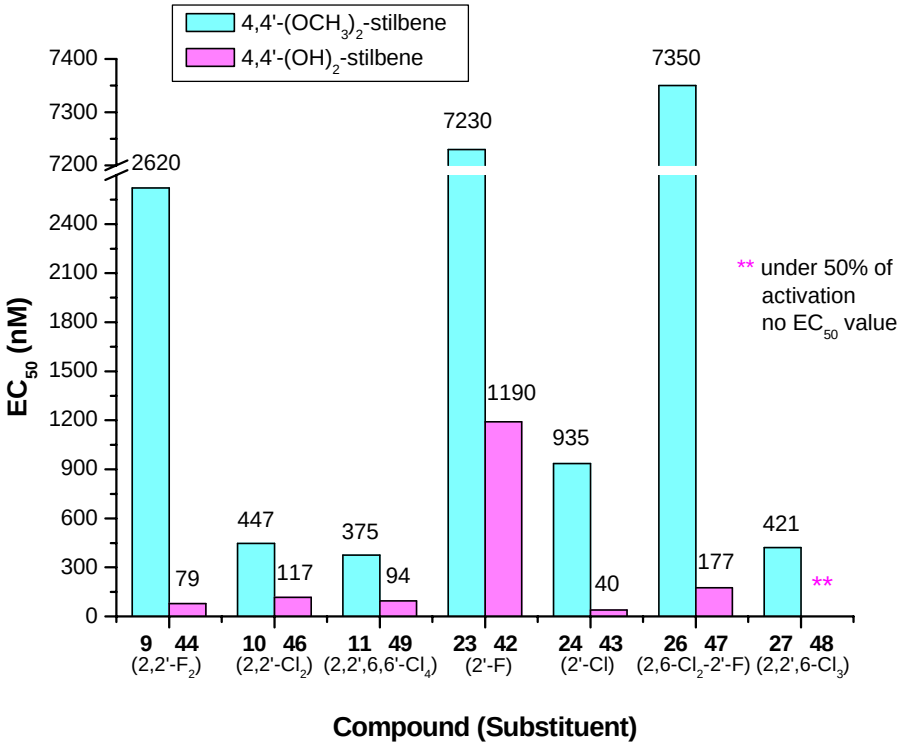



**Figure 38**. Comparison of the $EC_{50}$ values of the halo-substituted *E*-4,4'-$(OCH_3)_2$-stilbenes and *E*-4,4'-$(OH)_2$-stilbenes.

Each compared pair bears the same halo-substituents. No $EC_{50}$ value was given by **48**, because the relative gene activation was under 50% in the tested range of concentrations. This means that the $EC_{50}$ value by **48** should be higher than $10^{-5}$ M (10000 nM) if it exists. It is easy to find that all 4,4'-dihydroxystilbenes except **48** exhibited lower $EC_{50}$ value than the corresponding 4,4'-dimethoxystilbenes, that is, the former has greater agonist potency than the later. Nevertheless, this still does not point to the conclusion that 4,4'-dihydroxystilbenes are also better agonists than the corresponding 4-hydroxy-4'-methoxystilbenes. Which is the better agonist between the both types of hydroxylated stilbenes depends on other halo-substituents on the phenyl ring. Four groups of the compounds selected from three types of 4,4'-dimethoxystilbene, 4-hydroxy-4'-methoxystilbene and 4,4'-dihydroxystilbene separately with same halo-substituents in same group, and with different halo-substituents in different groups, are graphed in figure 39 to compare their $EC_{50}$ values. Like 4,4'-dihydroxystilbenes, four 4-hydroxy-4'-methoxystilbenes are also agonistically more active compared to the corresponding 4,4'-dimethoxystilbenes. Surprising is that, the 4-hydroxy-4'-methoxystilbenes **33** and **40** have lower $EC_{50}$ values than the 4,4'-dihydroxystilbenes **42** and **48**, while the 4-hydroxy-4'-methoxystilbenes **34** and **39** possess higher $EC_{50}$ values than the 4,4'-

dihydroxystilbenes **43** and **47**. This indicates that the 4'-methoxy and 4'-hydroxy groups have different influence on agonistic activities of 4-hydroxylated stilbenes with different parent structures in the MCF-7-2a cell line, but no clear superiority or regularity between the 4'-methoxy and 4'-hydroxy group in both types of hydroxylated stilbenes is observed concerning stimulation of estrogenic effect by the corresponding compounds. Therefore, both types of stilbenes might exhibit higher estrogenic effect, so long as the appropriate substituents are introduced into the phenyl ring.

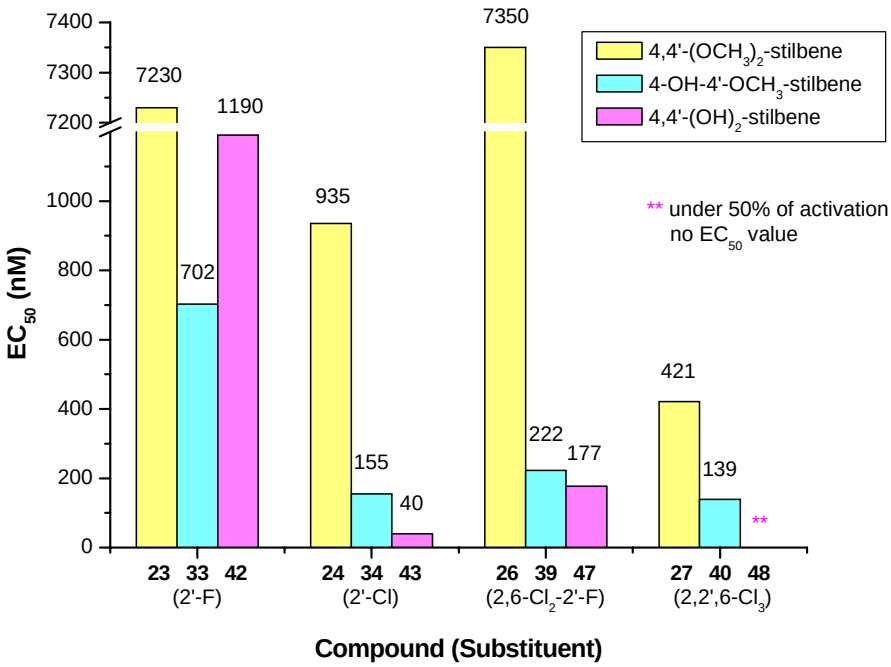



**Figure 39**. Comparison of the $EC_{50}$ values of the halo-substituted *E*-4,4'-$(OCH_3)_2$-stilbenes, *E*-4-OH-4'-$OCH_3$-stilbenes and *E*-4, 4'-$(OH)_2$-stilbenes.

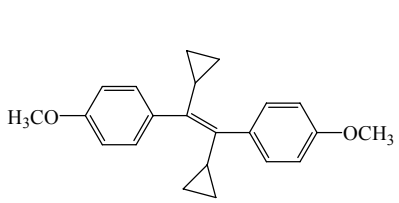
The analyses of crystal structures of ligand-ER LBD complexes discovered that a critical binding anchor of a ligand to the ER LBD is the phenolic hydroxy group that can bind to the Arg 394, Glu 353 and His 524 of the ER LBD as well as a water molecule through hydrogen bonds (see Section 1.4.1). A phenolic hydroxy group at para-position of the phenyl ring appeared to be essential for the estrogenic activity of stilbene derivatives [Sanoh, 86 and refs. cited therein]. For reasons given above in the present work a series of halo-substituted stilbene derivatives at least with a para-hydroxy group in one aromatic ring and a methoxy or hydroxy group in the other aromatic ring were tested for their estrogenic activities in MCF-7-2a cells. Like the well-known agonist DES, these stilbenes showed significant but different

agonistic effects. A para-methoxy or the second para-hydroxy group in stilbenes enhance the activities of stilbenes concerning ER-transcription. This can be explained by the structure similarities of the new stilbenes with DES and thus a DES-like interaction of the dihydroxylated stilbene with ER becomes reasonable. These stilbenes should belong to the type I estrogens that occupy the anchors at the ER similar to E2 and DES, as compared with the type II estrogens that are anchored in part to other amino acids at the ER [Gust, 174]. In previous reports, an ortho-chloro-substituent in the aromatic ring in the presence of a para-hydroxy group was proved to enhance the gene expression [Schertl, 92; Gust, 150]. In this work, ortho-chloro and para-methoxy substituents on one phenyl ring were also found to make the corresponding stilbene to a full-agonist as long as a para-hydroxy group was placed in another aromatic ring (e. g. stilbenes **34** and **40**). This may be attributed to the conjugate effect of stilbene molecules. This result provides not only new potential full-agonists but also a new line to develop active substances containing phenolic hydroxy and methoxy groups. In addition, symmetrically substituted stilbenes were demonstrated to be of great potency acting as full-agonists no matter which halo-substitutents are linked to the aromatic rings, e. g. **44** and **46**.

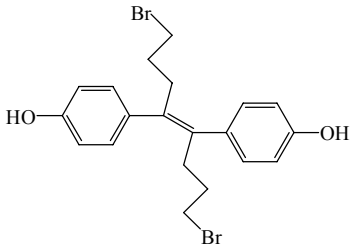
In conclusion, for halo-substituted (di)hydroxystilbenes — besides one para-phenolic hydroxy group in stilbenes, an other para-phenolic hydroxy or a methoxy group is of benefit to estrogenic activities of stilbenes. Appropriate halo-substituents linked to the aromatic ring of stilbenes enhance their estrogenic potency.

### 4.3.5 Tetrasubstituted ethenes containing at least two aryl-substituents

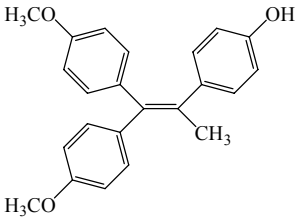
In previous reports, a series of 2-alkyl-1,1,2-triarylethenes were proved to be antagonistically active in the MCF-7-2a cell line. Some of them showed the same antagonistic potency as the strong antagonist 4-OH-tamoxifen [Lubczyk, 83 and 84]. In the present work, two DES analogues and several new 2-alkyl-1,1,2-triarylethenes were synthesized and investigated for their estrogenic properties in the MCF-7-2a cell line (see Table 15).



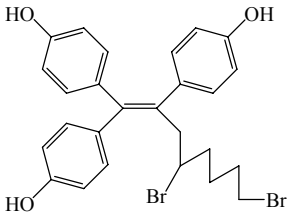

**54**



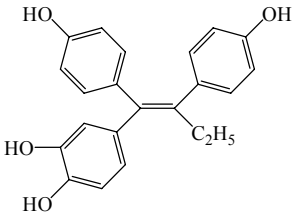

**55**



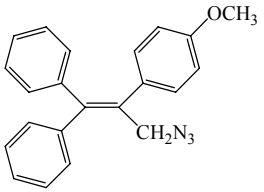

**76**




**79**




**80**




**99**

**Table 15**. Evaluation for Agonistic and Antagonistic Effects of Compounds **55**, **76**, **79**, **80** and **99** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1µM

| Compound | (*E* / *Z*) | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|
| | | $EC_{50}$ (nM) | 1µm (%) | $IC_{50}$ (nM) | 1µm (%) |
| **54** | *E* | - | 39.4 | - | 97.0 |
| **55** | *E* | 302 | 60.0 | - | 69.4 |
| **76** | - | - | -6.1 | 501 | 26.8 |
| **79** | - | - | 19.4 | 99 | 16.8 |
| **80** | *E* / *Z* | - | 5.1 | 514 | 38.7 |
| **99** | - | - | 28.0 | 5390 | 98.9 |
| **TAM** | | | | 500* | |
| **4-OHT** | | | | 7* | |

* [Lubczyk, 83 and 84]

The DES analogue **55** activated the luciferase expression with an $EC_{50}$ value of 302 nM and a relative activation of 60% at the concentration of 1 µM. The compound **54** showed a slight agonist activity. The other stilbene derivatives **76**, **79**, **80** and **99** induced no or less than 30% activation at the concentration of 1 µM.

Besides the significant estrogenic effect, the compound **55** showed also a slight inhibition of E2 with more than 50% remained activation in the range of tested concentrations, while the compound **54** was antagonistically inactive. The compound **79** antagonized completely the E2 effect at the higher concentration of 10 µM with an $IC_{50}$ value of 99 nM, which is better than

that of TAM (500 nM) and approaches that of 4-OHT (7 nM). The compound **76** has the same $IC_{50}$ value of 501 nM as that of TAM and also inhibits completely the E2-induced activation at the higher concentration of 10 µM (see Figure 40). The *E/Z*-isomer mixture **80** exhibited an inhibition of E2 to a remained activation of 38.7% at the concentration of 1 µM (22.8%, 10 µM) with an $IC_{50}$ value of 514 nM similar to that of TAM. The compound **99** showed a low antagonist activity with an $IC_{50}$ value of 5390 nM.

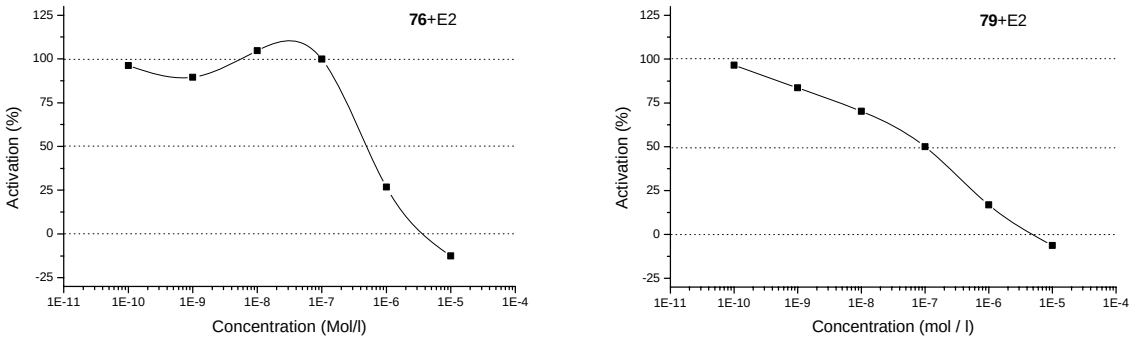



**Figure 40.** Antagonistic effects of **76** and **79** in luciferase assays with MCF-7-2a cells.

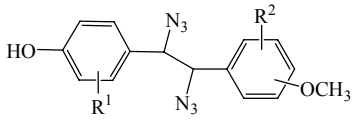
It is well-known that tamoxifen and its analogues functions as antagonists in breast tissues. The crystal analyses of 4-hydroxytamoxifen-ERα LBD complex demonstrated that the dimethylaminoethyl side chain of 4-hydroxytamoxifen protrudes and displaces H12 which interacts with the hydrophobic groove with a NR box-like sequence (LxxML versus LxxLL) functioning as an intramolecular mimic of the coactivator helix to prevent coactivator docking [Shiau, 75]. However, a series of 2-alkyl-1,1,2-triarylethenes without a bulky base side chain were proved to be antagonistically active in the MCF-7-2a cell line and some of them showed the same antagonistic potency as the strong antagonist 4-hydroxytamoxifen [Lubczyk, 83 and 84]. The antagonism of these compounds in ERα was postulated as a "passive antagonism", as was the case for THC in ERβ [Shiau, 76; see Section 1.4.1]. In the present work, all of the tested 2-alkyl-1,1,2-triarylethenes showed significant antagonistic effect in MCF-7-2a cells. The compounds **76** and **79** antagonized completely the E2 effect and the antiestrogenic effect of **79** was even higher than that of tamoxifen. Though none of them was such a strong antagonist as 4-hydroxytamoxifen, the results that all of them showed antagonistic activities as others described by Lubczyk et al. indicate that the skeleton structure of 2-alkyl-1,1,2-triarylethenes is one of the determining factors for hormonal properties in a certain tissue, while varieties of substituents including a base side are only enhancers. This skeleton determinant is also suitable for the other stilbene derivatives. For example, all of the stilbenes

**33 - 51** showed different agonist activities in the MCF-7-2a cell line without antagonistic effects.

### 4.3.6 Substituted 1,2-diazido-1-(4-hydroxyphenyl)-2-(4 or 6-methoxyphenyl)ethanes

1,2-Diaryl-1,2-diazidoethanes could be used as intermediates to synthesize some anticancer drugs containing the structure unit of 1,2-diarylethane [Gust, 94]. Some 1,2-diaryl-1,2-diazidoethanes were also proved to have agonistic effects in *vivo* [Gust, 93]. In the present work, we have synthesized a novel type of 1,2-diaryl-1,2-diazidoethanes bearing at least one hydroxy group on the phenyl ring. As primary tests, we used luciferase assays with MCF-7-2a cells to determine their potential agonistic and antagonistic activities.

The tested compounds **81 - 85** were evaluated by calculation of $EC_{50}$ / $IC_{50}$ values and relative activation at the concentration of 1μM (see Table 16).




**81 -85**

**Table 16**. Evaluation for Agonistic and Antagonistic Effects of Compounds **81 - 85** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1μM

| Compound | $R^1$ | $R^2$ | $OCH_3$ | Configuration | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $EC_{50}$ (nM) | 1μM (%) | $IC_{50}$ (nM) | 1μM (%) |
| **81** | H | 2-F | 4- | *threo* | - | 0.1 | - | 104.8 |
| **82** | H | 2-Cl | 4- | *threo* | - | 16.2 | - | 93.4 |
| **83** | H | 2,6-$Cl_2$ | 4- | *threo* | 4730 | 24.6 | - | 109.9 |
| **84** | 2,6-$Cl_2$ | H | 4- | *threo* | - | 26.9 | - | 124.6 |
| **85** | H | 2,4-$Cl_2$ | 6- | *threo* | - | 10.5 | - | 135.2 |

These results indicate that compounds **81 - 85** possess no or weak agonistic effects. Only **83** showed more than 50% activation in the range of tested concentrations and the maximum activation with a value of only 64% was exhibited at the concentration of 10 μM (Figure 41). Compounds bearing more chloro substituents appear to have stronger agonist activities. A

further comparison upon the influence of substituents on agonist activities of the compounds is of less interest due to the lower activation.

None of the five tested compounds showed an antagonistic effect in the MCF-7-2a cell line.

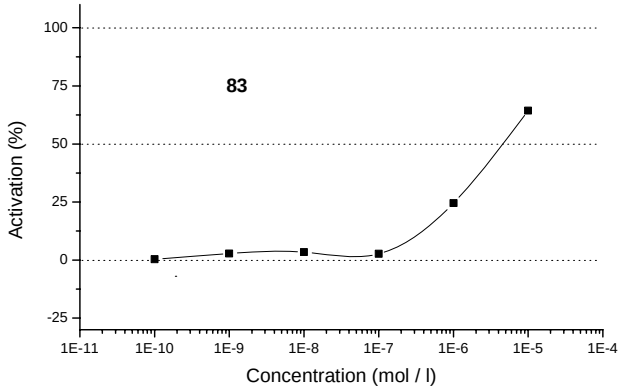
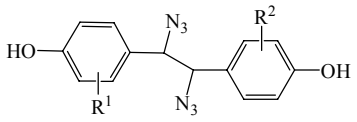



**Figure 41**. Agonistic effect of compound **83** in the MCF-7-2a cell line.

### 4.3.7 Substituted 1,2-diazido-1,2-bis(4-hydroxyphenyl)ethanes

The test results of 1,2-diazido-1,2-bis(4-hydroxyphenyl)ethanes **86** - **95** in the luciferase assay indicated that all of them showed significant agonist activities in the MCF-7-2a cell line with a maximal activation of more than 50% in the tested range of concentrations and thus each of them exhibited an $EC_{50}$ value (see Table 17).




**86 - 95**

**Table 17**. Evaluation for Agonistic and Antagonistic Effects of Compounds **86 - 95** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1μM

| Compound | $R^1$ | $R^2$ | Configuration | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|---|---|
| | | | | $EC_{50}$ (nM) | 1μM (%) | $IC_{50}$ (nM) | 1μM (%) |
| **86** | H | H | *meso* | 1810 | 31.3 | 626 | 49.0 |
| **87** | H | H | *dl* | 7340 | 35.8 | - | 69.2 |
| **88** | H | 2-F | *erythro* | 302 | 76.7 | - | 145.0 |
| **89** | H | 2-F | *threo* | 514 | 68.2 | - | 96.2 |
| **90** | 2-F | 2-F | *dl* | 198 | 78.9 | - | 80.6 |
| **91** | 2-F | 2-Cl | *erythro/threo* | 340 | 77.8 | - | 117.0 |
| **92** | 2-Cl | 2-Cl | *meso/dl* | 342 | 65.3 | - | 98.5 |
| **93** | 2-Cl | 2,6-$Cl_2$ | *erythro* | 65 | 101.0 | - | 97.0 |
| **94** | 2-Cl | 2,6-$Cl_2$ | *threo* | 199 | 61.1 | 9171 | 91.9 |
| **95** | 2,6-$Cl_2$ | 2,6-$Cl_2$ | *meso* | 56 | 77.6 | - | 106.8 |

Among these compounds, there are three pairs of diastereoisomers, *i. e.* **86** / **87**, **88** / **89** and **93** / **94** and two mixture of diastereoisomers, **91** and **92**. Each of **90** and **95** is only one of their two diastereoisomers. Comparing the $EC_{50}$ values of the three pairs of diastereoisomers, **86** (1810 nM) < **87** (7340 nM), **88** (302 nM) < **89** (514 nM), **93** (65 nM) < **94** (199 nM), one can easily find that the *erythro* (*meso*) diastereoisomer is a stronger agonist than the *threo* (*dl*). In addition, the agonistic activities of the tested 1,2-diaryl-1,2-diazidoethanes are clearly dependent on the halo-substituents. All of the halo-substituted 1,2-diazido-1,2-bis(4-hydroxy-phenyl)ethanes **88** - **95** have lower $EC_{50}$ values than the non-substituted **86** and **87**. The $EC_{50}$ values of **86** - **95** are collected into two groups according to their configuration and graphed in figure 42. It is easy to find that compounds having more halo-substituents induced higher agonist activities. This should be also supported by test results of two mixtures of diastereoisomers **90** and **91** containing two-fold halo-substituents, which have similar $EC_{50}$ values (340 and 342 nM) that are between 514 nM of the fluoro-substituted **89** and 199 nM of three-fold chloro-substituted **94**.

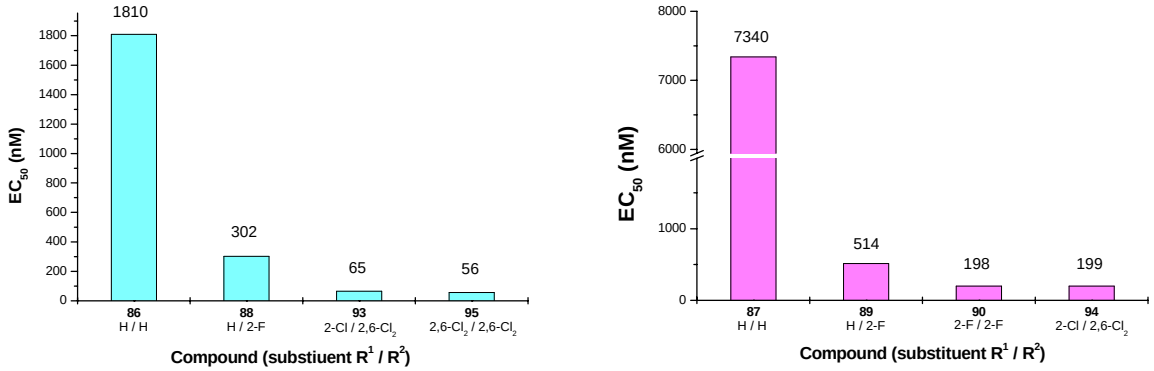



**Figure 42**. Influence of halo-substituents on $EC_{50}$ values of corresponding compounds.

Some of these diazidoethanes were full-agonists with a relative activation of 100% or more in the range of tested concentrations (see Figure 43, and 44 the first three graphic charts). Only **93** induced a maximum activation of 101% at the concentration of 1 µM, while the others, **86**, **89**, **90** and **95** reached their maximum activation of more than 100% at the concentration of 10 µM.

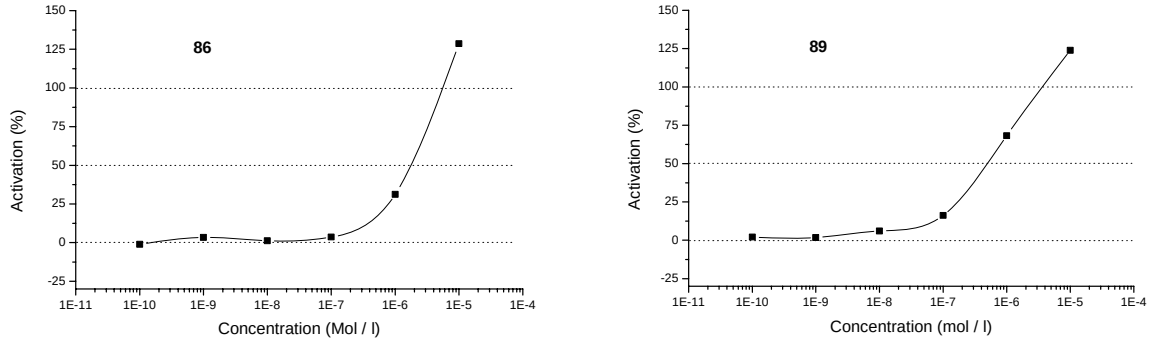



**Figure 43.** Agonistic effects of the compounds **86** and **89** in luciferase assays with MCF-7-2a cells.

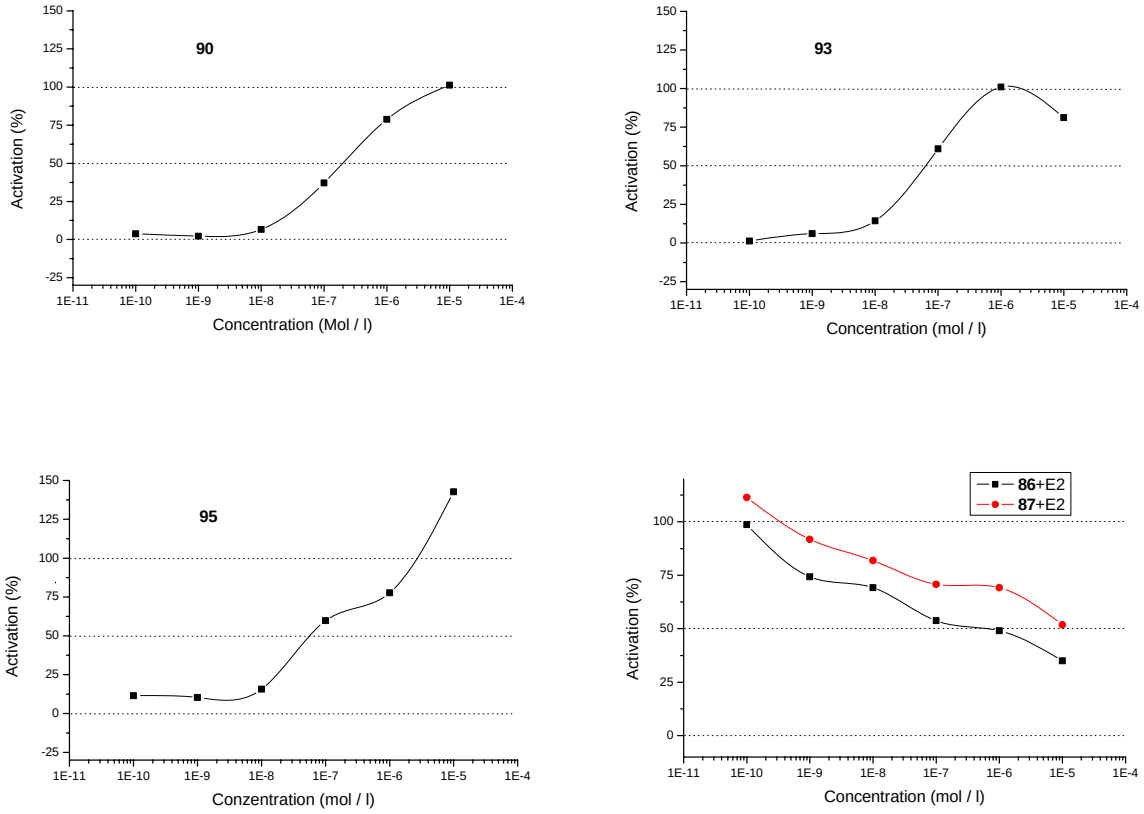



**Figure 44.** Agonistic effects of the compounds **90**, **93** and **95** as well as antagonistic effects of **86** and **87** in luciferase assays with MCF-7-2a cells.

Two of the tested diazidoethanes were antagonistically active in MCF-7-2a cells. The highest inhibition of 1 nM E2 was exhibited by the non-substituted compound **86** to a remained activation of 35% at the concentration of 10 µM with an $IC_{50}$ value of 626 nM (see Figure 44, the last graphic chart). At the concentration of 1 µM, only **86** and its diastereoisomer **87** had significant inhibition of E2 to 49.0% and 69.2% remained activation, respectively (see Table 17). The compounds **90**, **93** and **94** antagonized the E2 effect separately with a inhibition of E2 to 75.7%, 66.7% and 47.3% remained activation at the higher concentration of 10 µM.

Since that the unit of 1,2-diarylethane was introduced into the platinum complex to produce 1,2-diarylethylenediamine platinum (II) complexes (*meso*-1-ptLL') that possess antitumor and estrogenic effects [Gust, 26], this unit has been further conformationally constrained to heterocyclic rings, e. g. piperazine and imidazoline, and has led to a series of effective type II estrogens [Gust, 174 and 175; von Rauch, 176]. Chloro-substituted 1,2-bis(4-hydroxyphenyl)-ethanes have been also evaluated *in vivo* as potential anticancer compounds [Schertl, 92].

With such an effective pharmacophor, halo-substituted 1,2-diazido-1,2-bis(4-methoxyphenyl)ethanes have been demonstrated to have full-agonistic activities *in vivo* [Gust, 93]. Due to the critical role of the phenolic hydroxy group in hormonal anticancer drugs, hydroxylated 1,2-diaryl-1,2-diazidoethanes were expected to have higher biological activities than their methoxy-analogues. These known results prompted us to synthesize a series of the novel hydroxylated 1,2-diaryl-1,2-diazidoethanes and to study their biological properties. As expected, most of them were capable of activating transcription in MCF-7-2a cells and some of them were full-agonists, e. g. **90**, **93** and **95**. In contrast to the case of stilbene derivatives described in section 4.3.2 and 4.3.3, the estrogenic activities of the hydroxylated 1,2-diaryl-1,2-diazidoethanes showed a clear tendency dependent on the configuration of compounds and the substituents.

First, the *erythro* (*meso*) isomer is estrogenically more active than the *threo* (*dl*) isomer, this was demonstrated by all of the tested diastereoisomer pairs. Secondly, the dihydroxylated compounds are estrogenically more active than the monohydroxylated compounds bearing a methoxy group. The former showed more than 50% gene activation in the range of tested concentrations and some of them were full-agonist, while the later were not or only marginally active at the concentration of 10 μM. Being different to this, monohydroxylated stilbene with a para-methoxy group in the other aromatic ring may be full-agonist similar to dihydroxylated stilbenes. The different influence of hydroxy / methoxy groups on 1,2-diaryl-1,2-diazidoethanes from that on stilbene derivatives might be due to that the molecules of 1,2-diaryl-1,2-diazidoethanes are not conjugate. Thirdly, the hydroxylated 1,2-diaryl-1,2-diazidoethanes bearing more halo-substituents on aromatic rings showed greater estrogenic potencies. Fluoro-substituent enhances the agonist activity in the same way as chloro. Thus being consistent with this tendency, the most estrogenically active substance is the tetrachloro-dihydroxy-substituted *meso*-isomer **95**, and then followed by the trichloro-dihydroxy-substituted *erythro*-isomer **93**. The compound **93** already reached its maximum activation of 101% at the concentration of 1 μM. Due to the flexible and similar structure to that of DES, these effective compounds should belong to type I estrogens.

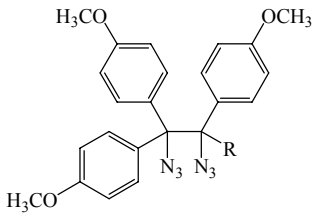
In addition, three of the hydroxylated 1,2-diaryl-1,2-diazidoethanes, *i. e.* **86**, **87** and **94** were antagonistically active in MCF-7-2a cells, but the antagonistic effects shown by them are low. Only the antagonistic effect shown by **86** is significant.

The above-described results arrive at the conclusion that halo-substituted 1,2-diazido-1,2-bis(4-hydroxyphenyl)ethanes, a novel family of 1,2-diazidoethanes, are new potential estrogens. The isomer *erythro*(or *meso*)-1,2-diazidoethane derivatives were agonistically more

active than their *threo*(or *dl*) isomers; The hydroxylated 1,2-diaryl-1,2-diazidoethanes bearing more halo-substituents on aromatic rings showed higher estrogenic potencies.

### 4.3.8 2-Alkyl-1,2-diazido-1,1,2-tris(4-methoxyphenyl)ethanes

Besides the above-described 1,2-diaryl-1,2-diazidoethanes, two novel tetrasubstituted 1,2-diazidoethanes **96** and **97** synthesized in this work were also tested for their estrogenic properties in the MCF-7-2a cell line (see Table 18).




**96, 97**

**Table 18**. Evaluation for Agonistic and Antagonistic Effects of Compounds **96** and **97** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1μM

| Compound | R | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|
| | | **$EC_{50}$ (nM)** | **1μm (%)** | **$IC_{50}$ (nM)** | **1μm (%)** |
| **96** | $CH_3$ | - | -5.0 | 1060 | 50.9 |
| **97** | $C_2H_5$ | - | 0.7 | - | 83.8 |

In contrast to 1,2-diaryl-1,2-diazidoethanes, tetrasubstituted 1,2-diazidoethanes **96** showed no agonistic effect but significantly inhibited the E2-induced activation in the luciferase assay with an $IC_{50}$ value of 1060 nM that is two times as great as that of TAM. At the substance concentration of 10 μM, the E2-induced activation remained only 18.1% (see Figure 45). The compound **97** showed neither agonistic effect nor significant inhibition of 1 nM E2. Only a slight inhibition was exhibited in the luciferase assay with a remained activation of 83.8% at the concentration of 1 μM ( 70.4%, 10 μM).

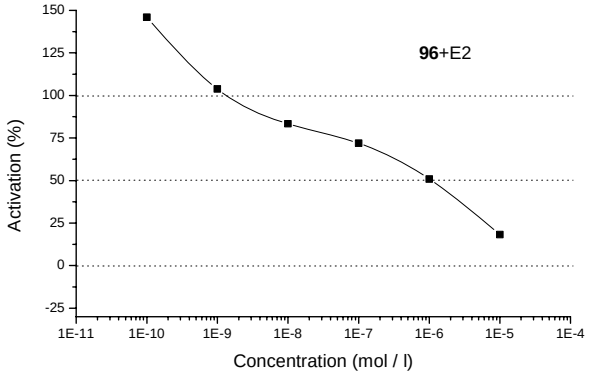
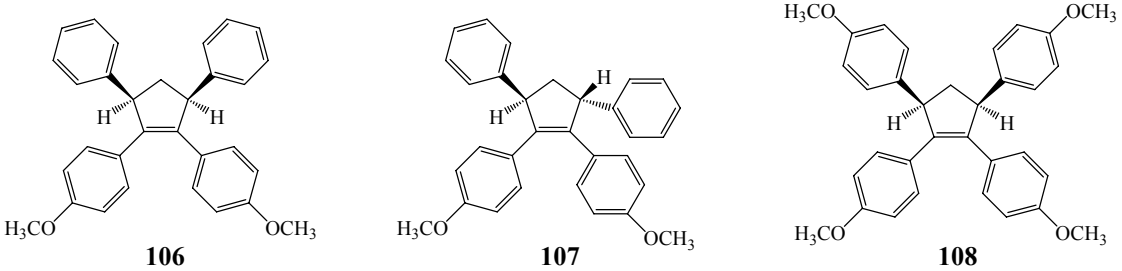



**Figure 45**. Antagonistic effect of compound **96** in the luciferase assay with MCF-7-2a cells.

Though the novel compound **96** showed a significant antagonist activity in MCF-7-2a cells, with which **96** can not yet be evacuated as a true antagonist. A possible reason for this lower activity maybe that the compound does not contain an antagonistically active substituent. If such an enhancer is introduced into the molecule, the produced compound will be expected to be a potential true antagonist. Therefore, this result is of more test interest to predict hormonal properties of this type compounds than the practical evaluation.

### 4.3.9 1,2,3,5-Tetraarylcyclopent-1-enes

1,2,3,5-Tetraarylcyclopent-1-enes **106** - **108** contain an unit of 4,4'-dimethoxystilbene. Attempts to obtain their 4,4'-dihydroxy derivatives were not successful. Therefore, only three compounds were tested for their estrogenic properties in the MCF-7-2a cell line (see Scheme 45 and Table 19).




**Scheme 45**

**Table 19**. Evaluation for Agonistic and Antagonistic Effects of Compounds **106 - 108** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1μM

| Compound | Configuration | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|
| | | $EC_{50}$ (nM) | 1μm (%) | $IC_{50}$ (nM) | 1μm (%) |
| **106** | *3R,5S / 3S,5R* | - | 16.8 | - | 101.4 |
| **107** | *3R,5R / 3S,5S* | - | 0.3 | - | 104.7 |
| **108** | *3R,5S / 3S,5R* | - | -9.5 | 9100 | 95.3 |

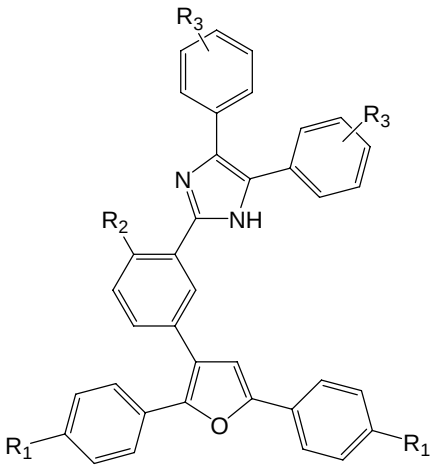
Unfortunately, they showed neither agonist nor antagonist activities in luciferase assays except the compound **108** that exhibited very low antagonist activity at the higher concentration of 10 μM with an $IC_{50}$ value of 9100 nM.

### 4.3.10 Triarylfurans containing a diarylimidazole unit

It is known that some triarylfurans can function as selective ligands for ERα [Mortensen, 146]. We introduced a diarylimidazole unit into an aryl ring of the triarylfuran to produce a novel compound skeleton. According to this procedure five triarylfurans containing diarylimidazole units **117 - 121** were synthesized and evaluated in luciferase assays to study their estrogenic / antiestrogenic properties. The results are sorted out in table 20.

The compounds **117** and **118** showed low but significant agonist activities with $EC_{50}$ values of 1450 and 5090 nM, respectively. At the concentration of 10 μM, the compound **117** induced a relative activation up to 92.4% (see Figure 46), while the compounds **119**, **120** and **121** exhibited no significant agonist activity.

The compound **120** showed low antagonist activity, but antagonized completely the E2 effect at higher concentration with an $IC_{50}$ value of 2750 nM (see Figure 46). The compound **121** reached its maximum inhibition of 1 nM E2 at the concentration of 1 μM with a remained activation of 57.1%, while **117 - 119** showed no significant antagonist activity.




**117 - 121**

**Table 20**. Evaluation for Agonistic and Antagonistic Effects of Compounds **117 - 121** by $EC_{50}$ / $IC_{50}$ and Relative Activation at the Concentration of 1µM

| Compound | $R^1$ | $R^2$ | $R^3$ | Agonistic effect | | Antagonistic effect | |
|---|---|---|---|---|---|---|---|
| | | | | $EC_{50}$ (nM) | 1µm (%) | $IC_{50}$ (nM) | 1µm (%) |
| **117** | 4-$OCH_3$ | 4-$OCH_3$ | 4-$OCH_3$ | 1450 | 46.1 | - | 100.5 |
| **118** | 4-$OCH_3$ | 4-$OCH_3$ | 4-F | 5090 | 33.6 | - | 89.8 |
| **119** | 4-$OCH_3$ | 4-$OCH_3$ | 2-Cl | - | 3.4 | - | 90.2 |
| **120** | 4-OH | 4-$OCH_3$ | 4-OH | - | 17.6 | 2750 | 86.5 |
| **121** | 4-OH | 4-OH | 4-OH | - | 19.4 | - | 57.1 |

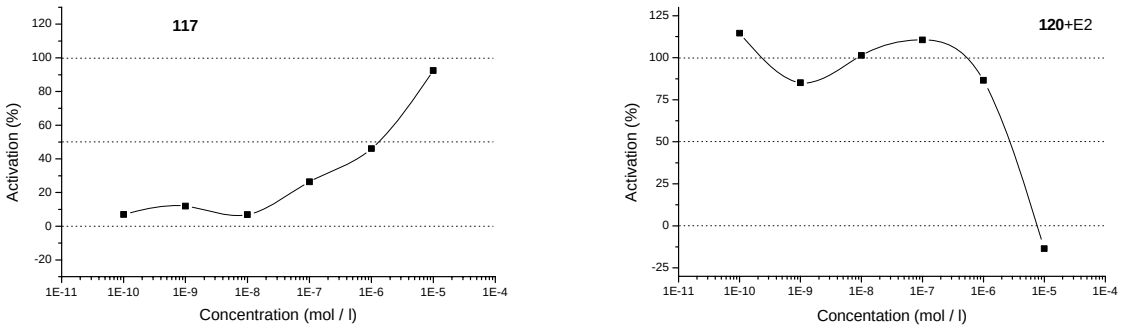



**Figure 46**. Agonistic effect of **117** and antagonistic effect of **120** in the MCF-7-2a cell line.

For an “active” antagonist, a bulky side chain plays an important role in its antagonism with precluding H12 from adopting the agonist-bound conformation, so as to alter the AF-2 structure and hinder coactivator docking (see Chapter 1.4.1). Hydroxylated triarylfuran derivatives have been found to have high ERα relative binding affinity (RBA) values where the affinity of E2 was set as 100% and to be fully efficacious on ERα in transcriptional activation assays [Mortensen, 146]. Therein the possible orientation of the triarylfurans in the ER ligand binding pocket has also been investigated using molecular modeling. In this work, diarylimidazole was introduced into the side phenyl ring of triarylfuran in order to examine whether this fascinate combination is beneficial to the transcriptional activity of the produced triarylfuran derivatives or the voluminous diarylimidazole component mimicking the so-called bulky side chain disorders H12 to produce an antagonistic effect. The lower hormonal activities of **117** - **121** in MCF-7-2a cells indicate that the diarylimidazole unit is neither an enhancer for the estrogenic activities of triarylfurans nor an antagonist promoter and the compounds **117** - **121** are not active enough to act as potential estrogen receptor modulators.

The results described in this chapter demonstrated a relationship between the hormonal properties and the structures of target compounds and simultaneously indicated that some of the novel (di)hydroxylated stilbenes and 1,2-diaryl-1,2-diazidoethanes have potentials to function as biological response modifiers, and several of the new 2-alkyl-1,1,2-triarylethenes and 2-alkyl-1,1,2-triaryl-1,2-diazidoethanes as antiestrogens for the treatment of breast cancer.

Agonistic / antagonistic effect is only one of the pharmacological evaluations. In order to totally evaluate the pharmacological potentials, further tests are necessary. It is also possible that these novel compounds are developed to treat other diseases.