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2-Phenyl-1-[4-(2-piperidine-1-yl-ethoxy)benzyl]-1*H*-benzimidazoles as ligands for the estrogen receptor: Synthesis and pharmacological evaluation

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ABSTRACT

2-Phenyl-1*H*-benzimidazoles **7a–e** were synthesized and tested for gene activation on ERα-positive MCF-7 breast cancer cells, stably transfected with the reporter plasmid ERE_{wtc}luc (MCF-7-2a cells). None of the compounds showed agonistic properties, but they antagonized dependent on hydroxyl groups at the benzimidazole core (5- or 6-OH) and at the aromatic ring in the 2-position (4-OH) in high concentrations the gene activation induced by estradiol (E2, 1 nM). All compounds exhibited significant antiproliferative properties on MCF-7 cells but they were inactive against hormone independent, ER negative MDA-MB-231 cells.

1. Introduction

Selective estrogen receptor modulators (SERMs) are small molecules whose pharmacological effects are caused by the binding to the estrogen receptors ERα and ERβ.[1,2] They possess tissue-specific agonistic or antagonistic properties. In bone and cardiovascular system, they are estrogenic, inhibiting postmenopausal bone loss and favourably influencing plasma lipoproteins and some coagulation factors. On the breast they have antagonistic effects.[3,4] Actually, there are two types of SERMs in clinical use. Based on their chemical structure they are classified to triarylethylenes and benzothiophenes.[5]

The prototype of SERM, (*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-*N*,*N*-dimethylethylamine (tamoxifen, Tam), was originally synthesized for the adjuvant treatment of breast cancer.[6] However, due to the partial estrogenic activity of the tamoxifen metabolites on the endometrium, the clinical use is associated with uterine hypertrophy and an increased risk of endometrial cancer. Further triarylethylene SERMs are droloxifene, toremifene, and idoxifene, also endowed with efficacy in the treatment of breast cancer.[7–10]

A representative of the benzothiophene type is raloxifene (Ral, see Scheme 1) which possesses a better toxicology profile and decreased endometrial estrogen agonism. It showed antagonistic effects in the breast similar to triphenylethylenes and no stimulatory effect in the endometrium.[11,12]

Another class of estrogens/antiestrogens include 1-{4-[2-(azepan-1-yl)ethoxy]benzyl}-2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (bazedoxifene see Scheme 1) which is now available in Europe for the treatment of osteoporosis.[13] Furthermore, related 2-phenylindoles derivatives synthesized by von Angerer and co-workers inhibited dependent on the substituents at the aromatic ring and the presence of a basic side chain ERα-mediated gene expression and inhibited estrogen-stimulated growth in estrogen-dependent breast cancer.[14,15]

Further substituted heterocycles, for example, furanes or pyrroles were optimized for the interaction with the ER.[16] In our group we selected as core molecules 2-imidazolines[17], piperazines[18] as well as imidazoles[19,20] and arranged aromatic rings at the heterocycle to achieve effective pharmacophores (Scheme 1).

While the 2,3-diarylpiperazines and the 4,5-diaryl-2-imidazolines were agonists at ERα, the imidazoles had to be triaryl-substituted to activate the ER molecule.[21,22] For example, the transcriptional activity of 5-ethyl-1,2,4-tris(4-hydroxyphenyl)-1*H*-imidazole (see Scheme 1) depended on the substituents at positions 4 and 5 as well as hydroxyl groups at the phenyl rings.

In continuation of this study we decided to use an OH-bearing benzimidazole core, meaning an exchange of the substituents at C4 and C5 of the imidazole by a fused aromatic ring. Furthermore, a structural assimilation to bazedoxifene was possible by using a benzyl instead of a phenyl ring at N1 together with an additional basic side chain at this ring (resulting in compounds **7a–e**).

In a theoretical study using Tripos Sybyl 6.7, we estimated the interaction of 2-phenyl-1*H*-benzimidazoles at the ligand binding

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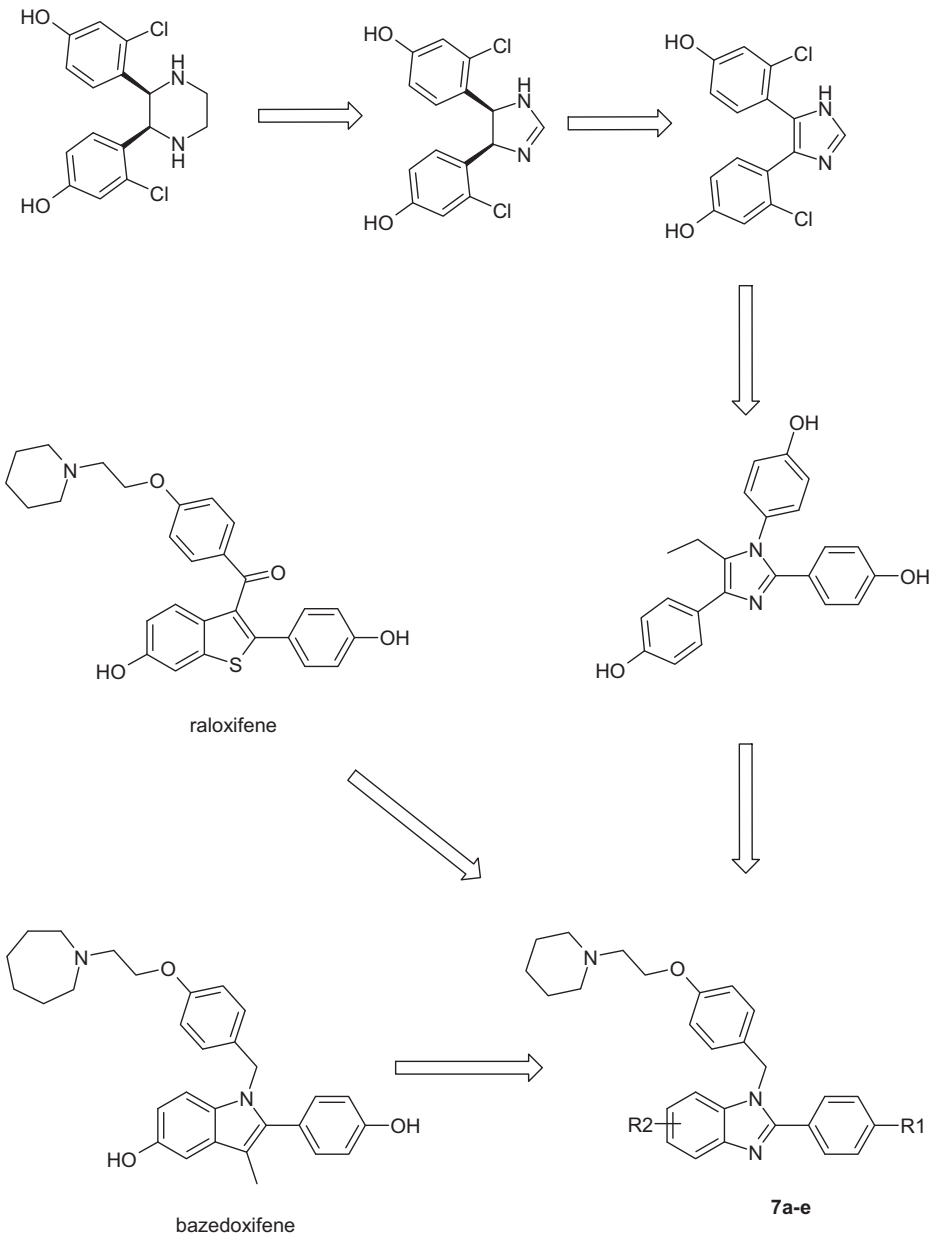


compd.	R_1	R_2	formula
7a	OH	H	$C_{27}H_{29}N_3O_2$
7b	H	5-OH	$C_{27}H_{29}N_3O_2$
7c	H	6-OH	$C_{27}H_{29}N_3O_2$
7d	OH	5-OH	$C_{27}H_{29}N_3O_3$
7e	OH	6-OH	$C_{27}H_{29}N_3O_3$

Scheme 1. Development of 2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazoles.

domain (LBD) of ERα on the example of **7d** and **7e**. We focused our attention to ERα, because the overexpression of this ER subtype is frequently observed in the early stage of breast cancer.[23]

The hydroxyl groups at **7d** and **7e** are arranged in a distance of 11.48 Å relevant for the attachment in the LBD of the ERα. The structures were energy minimized by MM3 and superimposed with Ral obtained from the X-ray structure of the co-crystallisate with ERα.[24] Both, the 5-OH (**7d**) and the 6-OH (**7e**) derivatives showed good similarity to Ral (Fig. 1) leading to the assumption of a Ral-like orientation in the LBD of ERα as shown for **7e** in Figure 2.

Contacts via hydrogen bonds of the hydroxyl groups at the 5- or the 6-position of the benzimidazole to Glu353 and Arg394 as well as a structurally conserved water molecule are possible, while the basic side chain is located in the side pocket building a salt bridge to the carboxylate group of Asp351 (Fig. 2). Finally, the 4-OH group at the 2-phenyl ring is bound to His524.

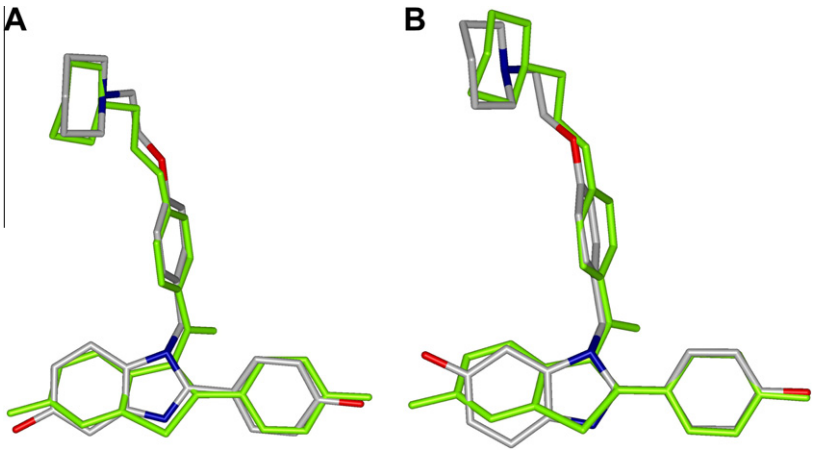


Figure 1. Superposition of the minimized structures of raloxifene and **7d** (A) or **7e** (B), respectively.

These positive results induced us to synthesize compounds **7a**–**e** to study the influence of the hydroxyl groups on hormone dependent ERα-positive MCF-7 cells.

2. Results

2.1. Chemistry

The 2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazoles were synthesized as illustrated in Scheme 2.

The reaction of the benzonitriles **1a**–**b** with thionyl chloride in ethanol yielded the benzimidic acid ethyl ester hydrochlorides **2a**–**b** which were subsequently reacted with the benzene-1,2-diamines **3a**–**b** in ethanol to obtain the 2-phenyl-1*H*-benzimidazoles **4a**–**c**.[25,26]

Compound **6a** or the mixture of the 5-(6)-methoxy isomers **6b**–**c** resulted from N-alkylation of the benzimidazoles **4a**–**c** by treatment with sodium hydride in dry DMF, followed by addition of **5**.[15,27] Ether cleavage with ethanethiol/$AlCl_3$ gave the target compounds **7a**–**e**. The separation of the 5- and 6-hydroxyl isomers was realized using MPLC on an RP-18 phase.

2.2. Biological properties

The benzimidazoles were tested in a HAP assay[28] for the ability to compete with [^{3}H]E_2 for binding at ERα or ERβ. Compound **7b** bound to ERα (RBA = 0.43%) and ERβ (RBA = 0.54%) with comparable affinity. A shift of the hydroxyl group from position 5 into position 6 strongly increased the affinity to ERα (RBA(**7c**) = 0.92%) and led to a complete loss of affinity to ERβ. Interestingly, the introduction of a further OH group in the 2-phenyl ring decreased the RBA value (RBA(**7d**) = 0.48%; RBA(**7e**) = 0.34%) but prevent the ERα-subtype selectivity. Furthermore, it should be noted that it is not inevitably necessary to have an OH group at the benzimidazole core. The 2-phenol derivative **7a** caused an RBA of 0.54%.

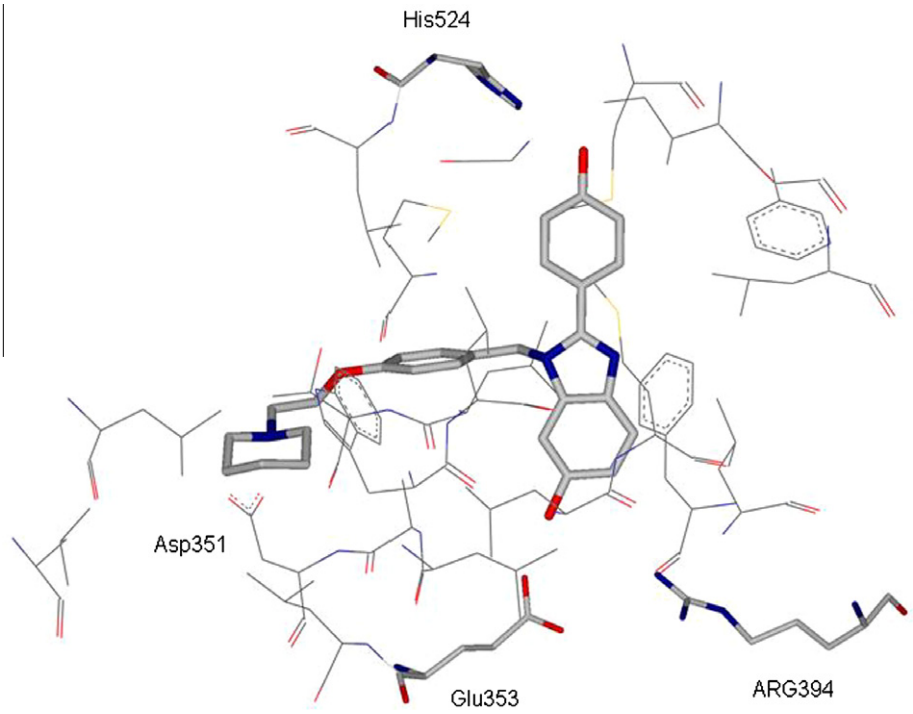
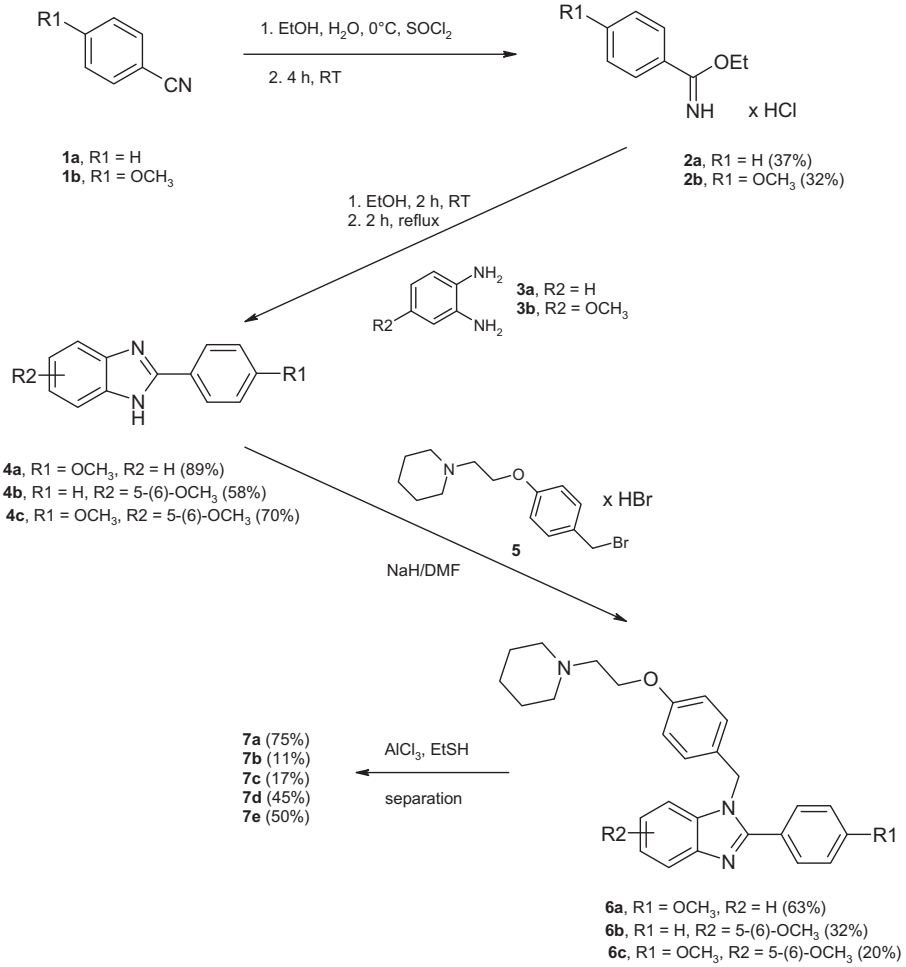


Figure 2. Theoretical binding mode of the 2-phenyl-1*H*-benzimidazole **7e** in the LBD of the ERα.



Scheme 2. Synthesis of 2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazoles.

The ERα-binding of **7a**–**e** was indeed lower than that of 4OHT (RBA = 2.7%) and Ral (RBA = 8.8%) but comparable to Tam (RBA = 0.6%). Thus, the benzimidazoles were evaluated for agonistic or antagonistic properties in a cell based assay using hormone dependent, ERα-positive MCF-7 breast cancer cells stably transfected with the plasmid ERE_{wtc}luc (MCF-7-2a cells).[29]

Because this assay requires transfer of the drugs through the cell membrane, it is necessary to confirm the cellular drug uptake. For this experiment the most affinic compound **7c** was a suitable candidate, because it showed emissions beyond the intrinsic fluorescence of tumor cells allowing fluorescence analysis (see Table 1 and Fig. S1, Supplementary data). The intracellular concentration of **7c** after an incubation time of 6 and 24 h amounted to 27 and 284 μM corresponding to a high accumulation grade of 2.7 and 28.4 in relation to the extracellular concentration of 10 μM.

In the cytosol of MCF-7-2a cells, drugs can interact with ERα. On the one hand, the binding of a hormonally active drug (agonist) to the ER leads to ER dimerization, binding to specific sequences of the reporter plasmid ERE_{wtc}luc and stimulation of the transcription of the luciferase gene. On the other hand, reduction of the E_2-stimulated luciferase expression points to antagonistic potency.[14]

Compound **7c** did not induce ER-mediated luciferase expression (data not shown), but completely antagonized the E_2 effect (concn 1 nM) at a concentration of 10 μM (IC_{50} = 0.31 μM). Its 5-OH isomer **7b** was less active with an IC_{50} = 1.31 μM (Fig. 3). Compound **7a** with a 4-OH group at the C2-phenyl ring reduced the E_2 effect at 10 μM to 31% (IC_{50} = 5.1 μM). Hydroxyl groups at both the benzimidazole core and the C2-phenyl ring did not increase the antagonistic potency. The IC_{50} values of **7d**–**e** amounted to 1.30 and 1.13 μM, respectively. Without having hormonal activity the antagonistic potency of **7a**–**e** was very similar to that of the reference compound Tam (IC_{50} = 0.88 μM). Compared with Ral (IC_{50} = 0.013 μM) and 4OHT (IC_{50} = 0.019 μM), all compounds were less active.

In the next step the antitumor potency of the benzimidazoles and the references were tested in vitro in a time-dependent chemosensitivity assay using hormone dependent MCF-7 and hormone independent MDA-MB-231 cells.[30,31]

Ral, Tam and 4OHT reduced both the growth of MCF-7 (Fig. 4) and MDA-MB-231 cells (data see[32]). They showed against the MCF-7 cell line cytostatic properties (T/C_{corr} = 0–20%) at a concentration of 5 μM but did not cause cytocidal effects at 10 μM.

In contrast, the benzimidazoles **7a**–**e** exhibited strong growth inhibition of MCF-7 cells (Fig. 5) and were completely inactive at the MDA-MB-231 cell line (data not shown).

Compounds **7a**–**c** caused cytocidal effects (T/C_{corr} <0%) at 10 μM, cytostatic effects ($T/C_{corr} \approx 0\%$) at 5 μM and reached the IC_{50} (T/C_{corr} = 50%) at 1 μM. A comparison of the graphs depicted in Figures 4 and 5 indicated a higher activity of **7a**–**c** compared to Ral, Tam and 4OHT. Compounds **7a** and **7b** were even more active than the established antitumor drug cisplatin (DDP, see Fig. 5).

Unexpected low antiproliferative effects caused **7d** and **7e** independent on the position of the OH group at the benzimidazole core. This might result from their interaction with the adenosine A_1 receptor expressed in MCF-7 cells, which influence the proliferation of MCF-7 cells.[33,34] Compounds **7a**–**e** were investigated for their affinity to adenosine A_1, A_{2A} and A_3 receptors using radio-ligand

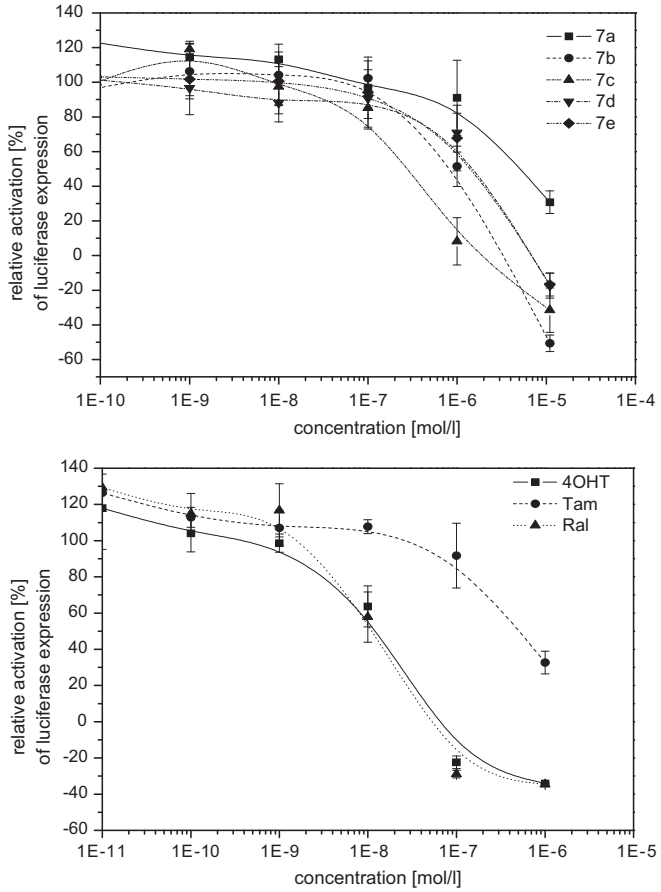


Figure 3. Luciferase expression (%) in MCF-7-2a cells treated with a combination of estradiol (1 nM) together with either the 2-phenyl-1*H*-benzimidazoles **7a**–**e** or the reference substances raloxifene (Ral), tamoxifen (Tam) and 4-hydroxytamoxifen (4OHT). Values expressed are the means ± SE of threefold determination in a single experiment.

binding assays. At a concentration of 10 μM only **7d**–**e** exhibited significant affinity for the A_1-R, and demonstrated selectivity for that receptor subtype (K_i values of about 3.7 ± 1.3 μM). The other compounds were inactive at all investigated adenosine receptor subtypes.

MCF-7 cells incubated with compounds **7a**–**e** drastically changed their morphology. As an example, the effects of compound **7a** are depicted in Figure 6.

Without drug, the cells grew as monolayer and were attached at the bottom of the cell culture flask with well-separated nucleus and cytoplasm (Fig. 6A). Already after an incubation time of 24 h dark deposition products around the nuclei appeared (Fig. 6B), which increased during the next 24 h (Fig. 6C). After 72 h the separation of nucleus and cytoplasm was lost and a strong cell fragmentation took place (Fig. 6D). Because Tam and Ral did not cause these effects, it is very likely that benzimidazoles and the reference substances possess different modes of action.

An explanation of the cell fragmentation might be DNA binding after accumulation in MCF-7 cells, which is already known from other benzimidazoles.[35,36] Intercalation between successive DNA base pairs, however, can be excluded because **7a**–**e** only marginally changed the melting point of ct-DNA in PBS (T_m = 64 °C) by $\Delta T_m \approx +3$ °C at a DNA base pair/ligand ratio of 0.4. Classical intercalators led already at a ratio of 0.1 to a considerably stronger shift. Nevertheless, a non-intercalative DNA interaction might be possible, since fluorescence spectroscopic investigations with the most cytotoxic compound **7a** demonstrated a strongly quenched fluorescence emission (Fig. 7) and a shift of the excitation wavelength by measurement in the presence of ct-DNA.[37] The same experiment with human serum albumin (HSA) in PBS led to additive fluorescence and indicated a specific binding to DNA which caused the fluorescence quenching.

3. Discussion

2-Phenyl-1-[4-(2-piperidin-1-yl-ethoxy)benzyl]-1*H*-benzimidazoles were synthesized as SERMs due to their structural similarity to Ral or bazedoxifene.

Bazedoxifene binds to both intracellular receptors with an around fourfold higher affinity for ERα. In MCF-7 cells transfected with an ERE-tk luciferase plasmid bazedoxifene showed no agonistic activity but inhibited the E_2 transactivation.[13]

The benzimidazole derivatives **7a**–**e** possess a comparable profile with high selectivity for ERα and potency to antagonize the E_2 effects (IC_{50} = 0.31–5.1 μM) in MCF-7 cells, however, to a lesser extent than bazedoxifene (IC_{50} = 3.7 nM) or Ral (IC_{50} = 13 nM).

They exhibit good structural similarity to Ral (see **7d**–**e**, Fig. 1) so that a comparable orientation in the LBD of ERα can be as-

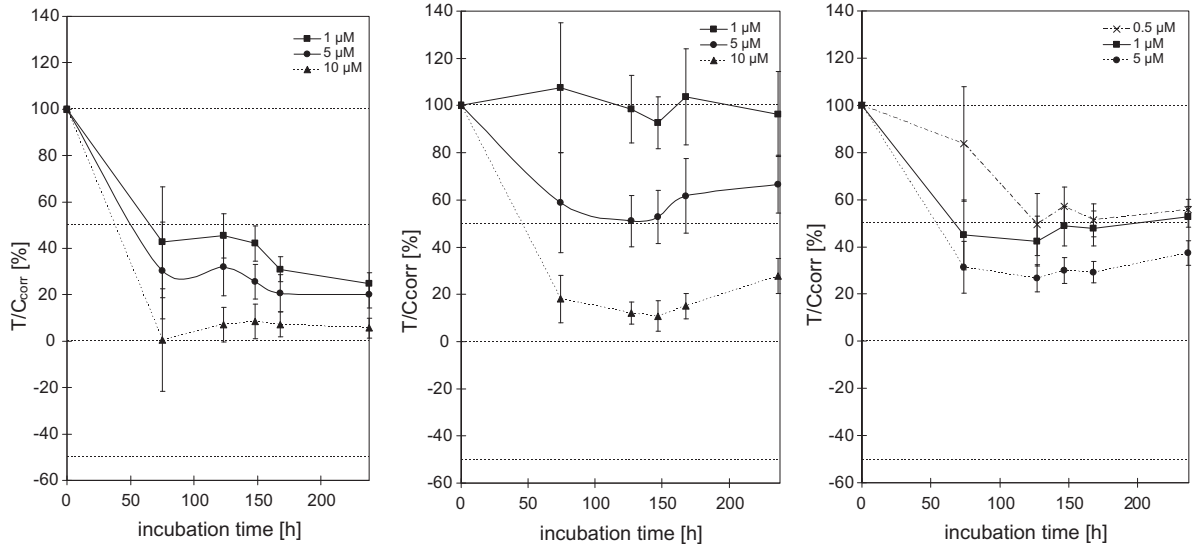


Figure 4. Cytotoxic effects of Ral (left), Tam (middle) and 4OHT (right) against MCF-7 cells.

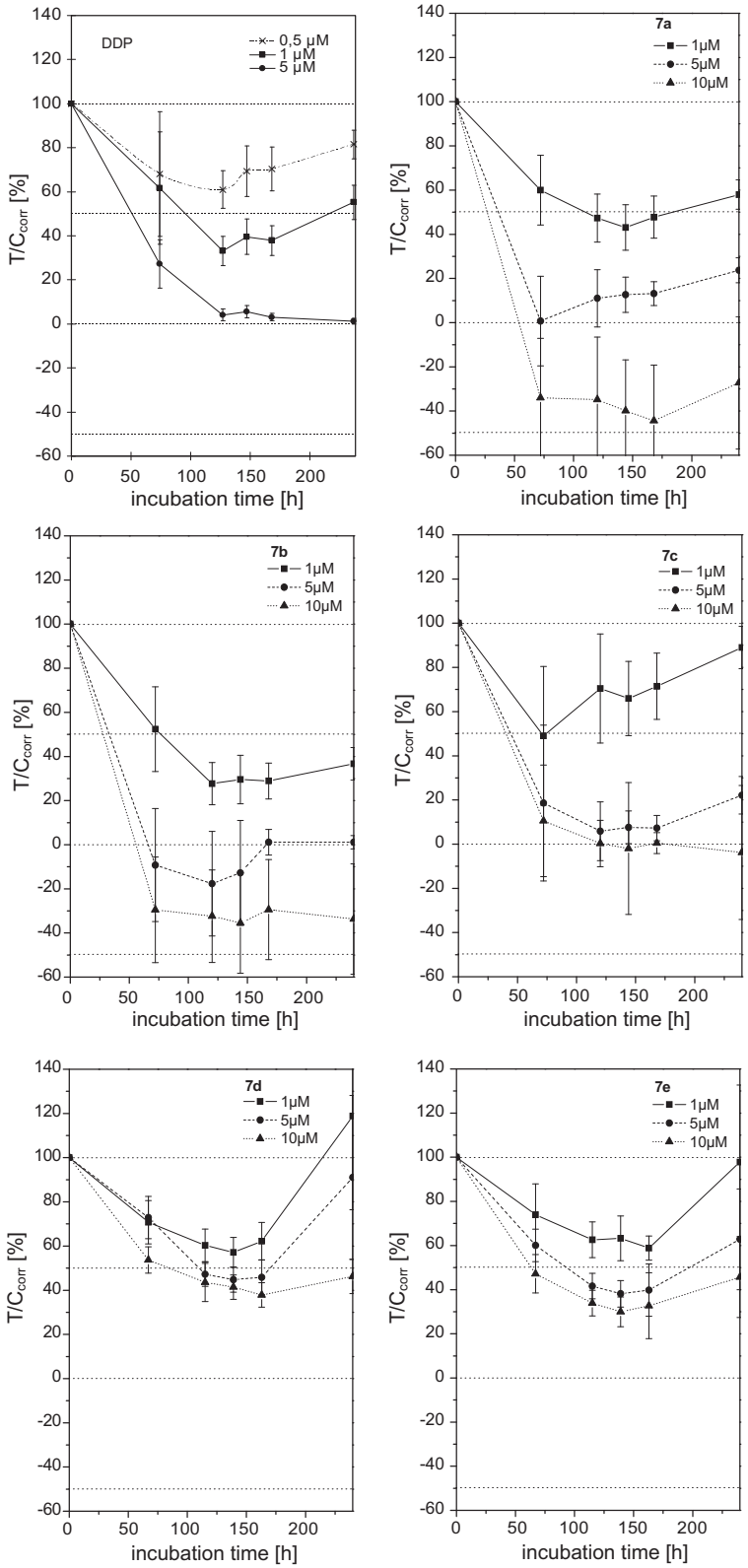


Figure 5. Growth curves of MCF-7 breast cancer cells in the presence of the 2-phenyl-1*H*-benzimidazole derivatives **7a**–**e** at concentrations of 1, 5 and 10 µM. Cisplatin (DDP) was used at concentrations of 0.5, 1 and 5 µM. Cell density was determined by crystal violet staining expressed as $T/C_{corr} = [(T - C_0)/(C - C_0)] \times 100$ [%] with T (test), C (control) and C_0 (cell extract immediately before compound treatment). Values expressed are the means ± SE of 16-fold determination in a single experiment.

sumed (see Fig. 2). The OH groups might interact with Glu353 and Arg394 (5-OH or 6-OH) as well as His524 (4′-OH). Both interactions at the same time, however, are not necessary for antiestrogenic effects.

The 2-(4-hydroxyphenyl)-1*H*-benzimidazole **7a** and the 5-(6)-hydroxy-2-phenyl-1*H*-benzimidazoles **7b**–**c** antagonized the E_2 effect at a concentration of 10 µM. It is very likely that **7a** adapts in the LBD a binding mode different from **7b**–**d**. On the one hand, the

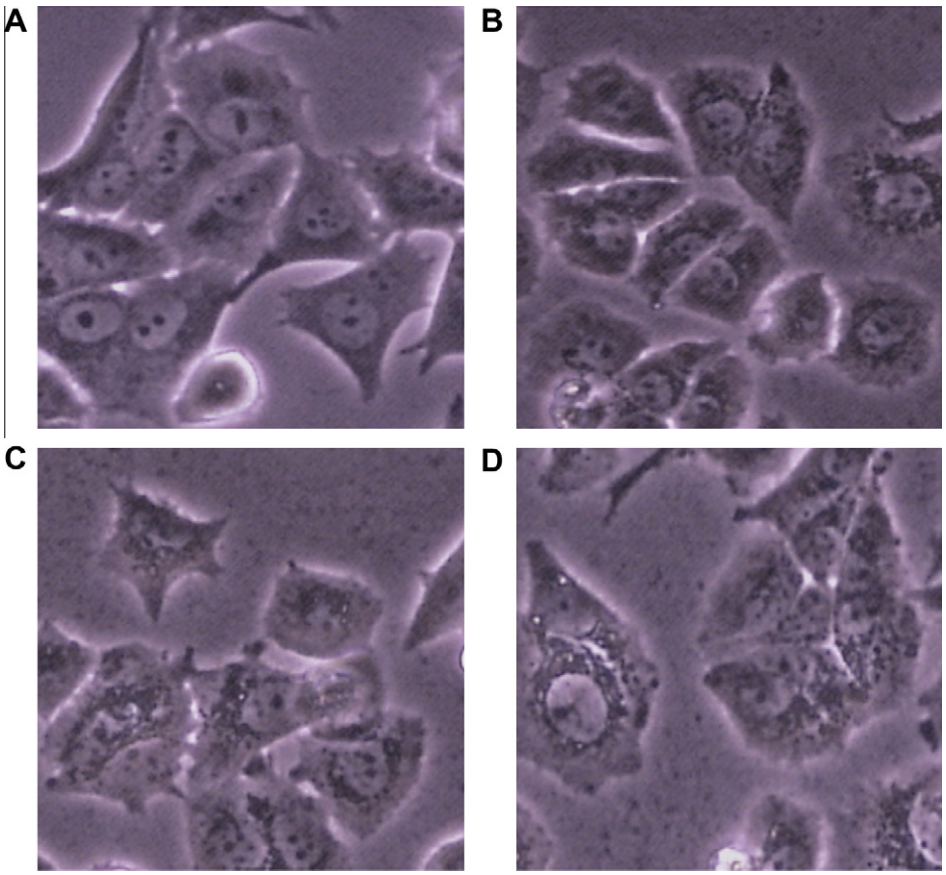


Figure 6. Influence of **7a** (concn: 10 μM) on MCF-7 cells. (A) Cells without substance. (B) Cells with compound **7a** after an incubation time of 24 h. (C) Cells with compound **7a** after an incubation time of 48 h. (D) Cells with compound **7a** after an incubation time of 72 h.

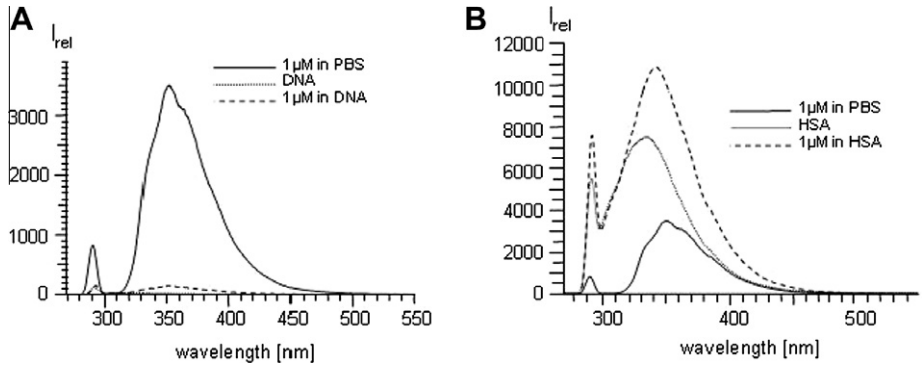


Figure 7. Fluorescence spectra of **7a** (1 μM): (A) in DNA-solution (1 mg/mL). (B) In HSA-solution (1 mg/mL).

C2-(4-hydroxyphenyl) ring of **7a** mimics the A-ring of E2 (binding to Glu353 and Arg394), while on the other hand the 5-(6)-hydroxybenzimidazole core of **7b**–**c** takes this orientation. The presence of a further OH group at the C2-phenyl ring of **7b**–**c** allowing additional interaction with His524 did not increase the antagonistic potency.

Similar observations were described by Grese et al. for a number of Ral analogues.[27,38] They showed that the 6-OH and, to a lesser extent, the 4′-OH substituents of Ral are important for receptor binding and in vitro activity. Furthermore, they proposed a contribution of the carbonyl 'hinge' between the basic amine-containing side chain and the core molecule.

A more consistent explanation for the lower antiestrogenic effects of **7a**–**e** compared to bazedoxifene might result from the second N-atom of the imidazole core. The higher hydrophilic character of the benzimidazole scaffold compared to indole and benzothiophene probably reduces the interactions with lipophilic amino acids in the LBD of ERα.

This obstacle can be overcome using a 2-Cl substituent in the C2-phenolic ring which protects the N-atom and increases the hydrophobicity of the molecule as we could already demonstrate for imidazolines and piperazines.[16–18]

It must be mentioned that the results of the luciferase assay with MCF-7-2a cells allow a statement to antiestrogenic properties only with restriction, because cytotoxic compounds reduce the cell number, resulting in a degradation of the gene activation curves.

All benzimidazoles (**7a**–**e**) were active in the time-dependent cytotoxicity test against MCF-7 cells. Compounds **7a**–**c** even

showed a profile comparable to the established antitumor drug cisplatin. The references Ral, Tam and 4OHT caused only cytostatic effects ($T/C_{corr} \approx 0$–20%) but showed no cytocidal properties. Furthermore, the benzimidazoles affected cytotoxicity selectively at MCF-7 cells. No antiproliferative effects were observed against MDA-MB-231 cells. The morphological change of MCF-7 cells as depicted in Figure 6 were not observed with Ral or 4OHT and pointed to a mode of action different from other SERMs.

4. Conclusion

2-Phenyl-1-[4-(2-piperidin-1-yl-ethoxy)benzyl]-1*H*-benzimidazoles represent a very interesting class of antitumor agents. They selectively damaged hormone dependent MCF-7 cells, while they were inactive against hormone independent MDA-MB-231 cells. They antagonized the effects of estradiol in hormone dependent MCF-7 cells and showed cytotoxic properties. The 5-hydroxy-2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (**7b**) was the most potent cytostatic and caused cytocidal effects already at a concentration of 5 μM.

5. Experimental section

5.1. General procedures

IR spectra (KBr pellets, film): Perkin–Elmer Model 580 A. ^{1}H NMR: ADX 400 spectrometer at 400 MHz (internal standard: TMS). EI-MS spectra: CH-7A-Varian MAT (70 eV). Elemental analyses: Microlaboratory of Freie Universität Berlin. MPLC-system (Knauer Smartline): HPLC pump 1000, HPLC-UV detector 2500, HPLC autosampler. As MPLC column a Eurospher 100-10 C18 250 × 16 mm id with Eurospher 100-10 C18 pre-column 30 × 16 mm id (Knauer) was used. All computational graphics were built using SYBYL 6.7 (Tripos Inc. 1699 South Hanley Road, St. Louis, MO 63144). Geometry optimization was carried out utilizing the MM3 force field within the program running on an INDY workstation.

5.2. Synthesis

Compound **5** was synthesized as described in Refs. 15,39.

5.2.1. General procedure for the synthesis of iminoether hydrochlorides

To a suspension of the respective nitrile (15 mmol) in 4 mL of ether, water (15 mmol) and ethanol (15 mmol) were added. The reaction mixture was stirred for 4 h at room temperature after thionyl chloride (15 mmol) was added slowly at 0 °C and was then combined with 70 mL ether. The pure product crystallized at −70 °C.

5.2.1.1. Benzimidic acid ethyl ester·HCl (2a). From **1a** (5.00 g, 48.49 mmol) and thionyl chloride (48.49 mmol). Yield: 3.35 g (37%), colorless solid. ^{1}H NMR (DMSO-d_6): δ = 1.49 (t, ^3J = 7.0 Hz, 3H, –CH_2–CH_3), 4.62 (q, ^3J = 7.0 Hz, 2H, –CH_2–CH_3), 7.66 (m, 2H, ArH), 7.83 (m, ArH), 8.08 (m, 2H, ArH), 11.11 (br s, 1H, NH_2), 11.88 (br s, 1H, NH_2). MS (EI, 130 °C): *m*/*z* (%) = 149 $[M]^{+\cdot}$ (7.1), 121 (40.0), 105 (100.0), 77 (48.6), 36 (26.1). IR (KBr): ν = 3089 (s), 2982 (s), 2926 (s), 1714 (m), 1606 (s), 1580 (s), 1506 (m), 1120 (m), 697 (s).

5.2.1.2. 4-Methoxybenzimidic acid ethyl ester·HCl (2b). From **1b** (2.00 g, 15.02 mmol) and thionyl chloride (15.02 mmol). Yield: 1.04 g (32%), colorless solid. ^{1}H NMR (DMSO-d_6): δ = 1.48 (t, ^3J = 7.0 Hz, 3H, –CH_2–CH_3), 3.88 (s, 3H, OCH_3), 4.59 (q, ^3J = 7.0 Hz, 2H, –CH_2–CH_3), 7.19 (d, ^3J = 9.0 Hz, 2H, ArH), 8.12 (d, ^3J = 9.0 Hz, 2H, ArH), 11.38 (br s, 2H, NH_2). MS (EI, 130 °C): *m*/*z* (%) = 179 $[M]^{+\cdot}$ (17.6), 151 (39.6), 135 (100.0), 36 (20.1). IR (KBr): ν = 3396 (w), 2997 (s), 2905 (s), 2842 (s), 1698 (m), 1603 (s), 1578 (s), 1524 (s), 1276 (s), 852 (m).

5.2.2. General procedure for the synthesis of 2-phenyl-1*H*-benzimidazoles

Amounts of 9.35 mmol of the appropriate benzimidic acid ethyl ester·HCl and the benzene-1,2-diamines were dissolved in 5 mL dry ethanol under N_2 atmosphere. The reaction mixture was stirred for 2 h at room temperature and heated to reflux for additionally 2 h. Subsequently, the solution was neutralized with 5% $NaHCO_3$, and the products were extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried over Na_2SO_4, and the solvent was evaporated. Purification was performed by column chromatography on silica gel with ether/petroleum ether 10:1.

5.2.2.1. 2-(4-Methoxyphenyl)-1*H*-benzimidazole (4a). From **2b** (261 mg, 1.21 mmol) and benzene-1,2-diamine **3a** (131 mg, 1.21 mmol). Yield: 242 mg (89%), colorless solid (mp 190–200 °C). ^{1}H NMR (DMSO-d_6): δ = 3.83 (s, 3H, OCH_3), 7.10 (d, ^3J = 8.8 Hz, 2H, ArH), 7.14–7.18 (m, 2H, ArH), 7.44–7.52 (m, 1H, ArH), 7.56 (m, 1H, ArH), 8.11 (d, ^3J = 8.8 Hz, 2H, ArH), 12.63 (s, 1H, NH). MS (EI, 60 °C): *m*/*z* (%) = 224 $[M]^{+\cdot}$ (100.0), 209 (28.8), 195 (8.1), 181 (15.1). IR (KBr): ν = 3411 (w), 3055 (m), 3007 (m), 2962 (m), 2929 (m), 2836 (m), 1612 (s), 1500 (s), 1255 (s), 838 (m), 746 (s).

5.2.2.2. 5-(6)-Methoxy-2-phenyl-1*H*-benzimidazole (4b). From **2a** (392 mg, 2.11 mmol) and 4-methoxybenzene-1,2-diamine **3b** (292 mg, 2.11 mmol). Yield: 275 mg (58%), colorless solid (mp 139 °C). Ratio of the isomeres 0.8:1. ^{1}H NMR (DMSO-d_6): δ = 3.80 (s, 2.4H, OCH_3), 3.82 (s, 3H, OCH_3), 6.82 (dd, ^3J = 8.9 Hz, ^4J = 2.5 Hz, 1H, ArH), 6.85 (dd, ^3J = 8.8 Hz, ^4J = 2.4 Hz, 0.8H, ArH), 6.99 (d, ^4J = 2.3 Hz, 1H, ArH), 7.19 (d, ^4J = 2.3 Hz, 0.8H, ArH), 7.40 (d, ^3J = 8.7 Hz, 0.8H, ArH), 7.43–7.58 (m, 6.4H, ArH), 8.03–8.19 (m, 3.6H, ArH), 12.73 (s, 1H, NH), 12.76 (s, 0.8H, NH). MS (EI, 100 °C): *m*/*z* (%) = 224 $[M]^{+\cdot}$ (100.0), 209 (68.7), 195 (11.0), 181 (15.7). IR (KBr): ν = 3411 (w), 3061 (m), 2920 (s), 2849 (m), 1631 (m), 1594 (w), 1270 (m), 810 (m), 695 (m).

5.2.2.3. 5-(6)-Methoxy-2-(4-methoxyphenyl)-1*H*-benzimidazole (4c). From **2b** (242 mg, 1.12 mmol) and 4-methoxybenzene-1,2-diamine **3b** (155 mg, 1.12 mmol). Yield: 200 mg (70%), colorless solid (mp 187 °C). Ratio of the isomeres 0.8:1. ^{1}H NMR (DMSO-d_6): δ = 3.78 (s, 2.4H, OCH_3), 3.80 (s, 3H, OCH_3), 3.86 (s, 5.4H, OCH_3), 6.79 (dd, ^3J = 9.0 Hz, ^4J = 2.4 Hz, 1H, ArH), 6.81 (dd, ^3J = 9.0 Hz, ^4J = 2.4 Hz, 0.8H, ArH), 6.95 (d, ^4J = 2.4 Hz, 1H, ArH), 7.08 (d, ^3J = 8.9 Hz, 2H, ArH), 7.09 (d, ^3J = 8.9 Hz, 1.6H, ArH), 7.15 (d, ^4J = 2.2 Hz, 0.8H, ArH), 7.36 (d, ^3J = 8.7 Hz, 0.8H, ArH), 7.49 (d, ^3J = 8.8 Hz, 1H, ArH), 8.05 (pt, ^3J = 8.8 Hz, ^3J = 8.9 Hz, 3.6H, ArH), 12.55 (s, 1H, NH), 12.56 (s, 0.8H, NH). MS (EI, 60 °C): *m*/*z* (%) = 254 $[M]^{+\cdot}$ (100.0), 239 (65.8), 211 (14.3), 196 (6.3), 168 (3.7). IR (KBr): ν = 3411 (m), 3052 (m), 3002 (m), 2924 (m), 2849 (m), 1635 (m), 1611 (s), 1581 (w), 1261 (s), 835 (m).

5.2.3. General procedure for the synthesis of 2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazoles

An amount of 0.80 mmol of the appropriate 2-phenyl-1*H*-benzimidazole was dissolved in 5 mL of dry DMF and cooled to 0 °C. NaH (2.94 mmol, 60% dispersion in mineral oil) was added and the mixture was stirred for 30 min. In a parallel reaction 4-(2-piperidin-1-yl-ethoxy)benzyl bromide hydrobromide **5** (1.60 mmol) in 5 mL dry DMF were allowed to react with NaH (5.88 mmol, 60% dispersion in mineral oil) at 0 °C for 30 min. Additionally, both mixtures

were combined, stirred for 2 h at 0 °C and further 4 h at room temperature. The reaction mixture was poured into water (20 mL), extracted with CH_2Cl_2 (3 × 50 mL), dried over Na_2SO_4, filtered and then the solvent was evaporated. The product was purified by column chromatography on silica gel with CH_2Cl_2/MeOH 98:2.

5.2.3.1. 2-(4-Methoxyphenyl)-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (6a). From **4a** (128 mg, 0.57 mmol). Yield: 160 mg (63%), yellow oil. ^{1}H NMR (DMSO-d_6): δ = 1.30–1.38 (m, 2H, CH_2), 1.38–1.55 (m, 4H, CH_2), 2.35–2.41 (m, 4H, CH_2), 2.54–2.61 (m, 2H, CH_2), 3.82 (s, 3H, OCH_3), 3.97 (t, ^3J = 5.9 Hz, 2H, –O–CH_2–), 5.47 (s, 2H, phenyl-CH_2–), 6.84 (d, ^3J = 8.6 Hz, 2H, ArH), 6.92 (d, ^3J = 8.6 Hz, 2H, ArH), 7.08 (d, ^3J = 8.7 Hz, 2H, ArH), 7.17–7.25 (m, 2H, ArH), 7.38–7.48 (m, 1H, ArH), 7.62–7.72 (m, 3H, ArH). MS (EI, 135 °C): *m*/*z* (%) = 441 $[M]^{+\cdot}$ (8.5), 411 (1.0), 343 (0.4), 329 (0.3), 251 (0.2), 217 (1.1), 113 (4.2), 98 (100.0). IR (film): ν = 3005 (w), 2934 (m), 2854 (w), 1674 (w), 1612 (m), 1580 (w), 1512 (m), 1251 (s), 837 (m), 750 (s).

5.2.3.2. 5-(6)-Methoxy-2-phenyl-1-[4-(2-piperidin-1-ylethoxy)-benzyl]-1*H*-benzimidazole (6b). From **4b** (239 mg, 1.07 mmol). Yield: 150 mg (32%), yellow oil. Ratio of the isomeres 0.8:1. ^{1}H NMR (DMSO-d_6): δ = 1.27–1.40 (m, 3.6H, CH_2), 1.40–1.52 (m, 7.2H, CH_2), 2.34–2.42 (m, 7.2H, CH_2), 2.58 (t, ^3J = 5.9 Hz, 3.6H, =N–CH_2–), 3.75 (s, 3H, OCH_3), 3.80 (s, 2.4H, OCH_3), 3.97 (t, ^3J = 6.0 Hz, 3.6H, –O–CH_2–), 5.46 (s, 1.6H, phenyl-CH_2–), 5.48 (s, 2H, phenyl-CH_2–), 6.79–6.85 (d, ^3J = 8.7 Hz, 3.6H, ArH), 6.85–6.95 (m, 5.4H, ArH), 7.04 (d, ^4J = 2.3 Hz, 1H, ArH), 7.23 (d, ^4J = 2.3 Hz, 0.8H, ArH), 7.35 (d, ^3J = 8.9 Hz, 0.8H, ArH), 7.41–7.56 (m, 5.4H, ArH), 7.60 (d, ^3J = 8.8 Hz, 1H, ArH), 7.64–7.76 (m, 3.6H, ArH). MS (EI, 320 °C): *m*/*z* (%) = 442 $[M]^{+\cdot}$ (4.7), 343 (0.3), 330 (0.2), 113 (0.4), 98 (100.0). IR (film): ν = 3431 (s), 3033 (w), 2924 (m), 2853 (w), 1616 (m), 1512 (w), 1243 (w), 818 (w), 743 (w), 697 (w).

5.2.3.3. 5-(6)-Methoxy-2-(4-methoxyphenyl)-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (6c). From **4c** (250 mg, 0.98 mmol). Yield: 94 mg (20%), colorless oil. Ratio of the isomeres 0.8:1. ^{1}H NMR (DMSO-d_6): δ = 1.30–1.40 (m, 3.6H, CH_2), 1.40–1.53 (m, 7.2H, CH_2), 2.35–2.43 (m, 7.2H, CH_2), 2.54–2.64 (m, 3.6H, =N–CH_2–), 3.74 (s, 2.4H, OCH_3), 3.79 (s, 3H, OCH_3), 3.81 (s, 2.4H, OCH_3), 3.82 (s, 3H, OCH_3), 3.91–4.11 (m, 3.6H, –O–CH_2–), 5.44 (s, 2H, phenyl-CH_2–), 5.45 (s, 1.6H, phenyl-CH_2–), 6.78–6.89 (m, 5.4H, ArH), 6.91 (d, ^3J = 8.6 Hz, 3.6H, ArH), 7.00 (d, ^4J = 2.4 Hz, 0.8H, ArH), 7.07 (d, ^3J = 8.7 Hz, 3.6H, ArH), 7.20 (d, ^4J = 2.4 Hz, 1H, ArH), 7.31 (d, ^3J = 8.8 Hz, 1H, ArH), 7.55 (d, ^3J = 8.8 Hz, 0.8H, ArH), 7.63 (d, ^3J = 8.8 Hz, 1.6H, ArH), 7.67 (d, ^3J = 8.8 Hz, 2H, ArH). MS (EI, 135 °C): *m*/*z* (%) = 472 $[M]^{+\cdot}$ (33.0), 254 (7.2), 113 (5.4), 98 (100.0). IR (film): ν = 3005 (w), 2934 (m), 2854 (w), 1674 (w), 1612 (m), 1580 (w), 1512 (m), 1251 (s), 837 (m), 750 (s).

5.2.4. General procedure for the ether cleavage with $AlCl_3$ and ethanethiole

A solution of 1*H*-benzimidazole (1 mmol) in 15 mL of dry CH_2Cl_2 was cooled to 0 °C. Then $AlCl_3$ (2 mmol) and ethanethiole (3 mmol) were added under N_2 atmosphere. The reaction mixture was stirred for 30 min under cooling and further 30 min at room temperature. The excess of $AlCl_3$ was hydrolyzed by addition of 10 mL water. After neutralization using 5% $NaHCO_3$ the phenolic product was extracted with CH_2Cl_2 (3 × 50 mL), the organic layers were combined, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure.

5.2.4.1. 2-(4-Hydroxyphenyl)-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (7a). From **6a** (106 mg, 0.24 mmol). Column chromatography on silica gel with CH_2Cl_2/MeOH 9:1. Yield: 77 mg (75%), colorless solid (mp 102 °C). ^{1}H NMR (DMSO-d_6): δ = 1.26–1.39 (m, 2H, CH_2), 1.40–1.59 (m, 4H, CH_2), 2.34–2.42 (m, 4H, CH_2), 2.54–2.63 (m, 2H, =N–CH_2–), 3.86–4.07 (m, 2H, –O–CH_2–), 5.46 (s, 2H, phenyl-CH_2–), 6.85 (d, ^3J = 8.6 Hz, 2H, ArH), 6.89 (d, ^3J = 8.7 Hz, 2H, ArH), 6.92 (d, ^3J = 8.6 Hz, 2H, ArH), 7.09–7.25 (m, 2H, ArH), 7.34–7.47 (m, 1H, ArH), 7.55 (d, ^3J = 8.5 Hz, 2H, ArH), 7.62–7.70 (m, 1H, ArH), 9.95 (s, 1H, OH). MS (EI, 330 °C): *m*/*z* (%) = 427 $[M]^{+\cdot}$ (8.4), 329 (0.2), 316 (0.8), 210 (4.7), 113 (0.5), 98 (100.0). IR (KBr): ν = 3426 (m), 3056 (m), 2933 (s), 1611 (s), 840 (w), 746 (m). Anal. Calcd for $C_{27}H_{29}N_3O_2$: C, 75.85; H, 6.84; N, 9.83. Found: C, 75.87; H, 6.85; N, 9.84.

5.2.4.2. 5-Hydroxy-2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (7b). From **6b** (150 mg, 0.34 mmol). Yield: 16 mg (11%), colorless oil. ^{1}H NMR (DMSO-d_6): δ = 1.27–1.41 (m, 2H, CH_2), 1.41–1.55 (m, 4H, CH_2), 2.34–2.42 (m, 4H, CH_2), 2.58–2.65 (m, 2H, =N–CH_2–), 3.85–4.05 (m, 2H, –O–CH_2–), 5.40 (s, 2H, phenyl-CH_2–), 6.72 (dd, ^3J = 8.6 Hz, ^4J = 2.0 Hz, 1H, ArH), 6.82 (d, ^3J = 8.5 Hz, 2H, ArH), 6.90 (d, ^3J = 8.5 Hz, 2H, ArH), 7.01 (d, ^4J = 1.8 Hz, 1H, ArH), 7.24 (d, ^3J = 8.7 Hz, 1H, ArH), 7.45–7.57 (m, 3H, ArH), 7.65–7.75 (m, 2H, ArH), 9.09 (s, 1H, OH). MS (EI, 270 °C): *m*/*z* (%) = 427 $[M]^{+\cdot}$ (5.8), 329 (3.1), 210 (1.7), 113 (0.6), 98 (100.0). IR (film): ν = 3435 (s), 2927 (m), 1616 (w), 1512 (w), 802 (w), 775 (w), 699 (w). Anal. Calcd for $C_{27}H_{29}N_3O_2$: C, 75.85; H, 6.84; N, 9.83. Found: C, 75.79; H, 6.85; N, 9.84.

5.2.4.3. 6-Hydroxy-2-phenyl-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (7c). From **6b** (150 mg, 0.34 mmol). Yield: 25 mg (17%), colorless solid (mp 94.5 °C). ^{1}H NMR (DMSO-d_6): δ = 1.28–1.41 (m, 2H, CH_2), 1.41–1.52 (m, 4H, CH_2), 2.34–2.43 (m, 4H, CH_2), 2.60 (t, ^3J = 5.9 Hz, 2H, =N–CH_2–), 3.97 (t, ^3J = 5.9 Hz, 2H, –O–CH_2–), 5.36 (s, 2H, phenyl-CH_2–), 6.68 (d, ^4J = 2.0 Hz, 1H, ArH), 6.73 (dd, ^3J = 8.7 Hz, ^4J = 2.2 Hz, 1H, ArH), 6.86 (d, ^3J = 8.7 Hz, 2H, ArH), 6.93 (d, ^3J = 8.7 Hz, 2H, ArH), 7.44–7.48 (m, 4H, ArH), 7.62–7.73 (m, 2H, ArH), 9.28 (s, 1H, OH). MS (EI, 270 °C): *m*/*z* (%) = 427 $[M]^{+\cdot}$ (5.8), 329 (3.1), 210 (1.7), 113 (0.6), 98 (100.0). IR (KBr): ν = 3400 (m), 3065 (m), 3031 (m), 2935 (s), 1620 (m), 1512 (s), 1246 (s), 824 (m), 754 (m), 700 (m). Anal. Calcd for $C_{27}H_{29}N_3O_2$: C, 75.85; H, 6.84; N, 9.83. Found: C, 75.95; H, 6.75; N, 9.81.

5.2.4.4. 5-Hydroxy-2-(4-hydroxyphenyl)-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (7d). From **6c** (263 mg, 0.56 mmol). Yield: 112 mg (45%), colorless oil. ^{1}H NMR (DMSO-d_6): δ = 1.31–1.41 (m, 2H, CH_2), 1.41–1.52 (m, 4H, CH_2), 2.34–2.42 (m, 4H, CH_2), 2.61 (t, ^3J = 5.7 Hz, 2H, =N–CH_2–), 3.98 (t, ^3J = 5.9 Hz, 2H, –O–CH_2–), 5.38 (s, 2H, phenyl-CH_2–), 6.63 (dd, ^3J = 8.6 Hz, ^4J = 2.1 Hz, 1H, ArH), 6.84 (d, ^3J = 8.7 Hz, 2H, ArH), 6.87 (d, ^3J = 8.6 Hz, 2H, ArH), 6.91 (d, ^3J = 8.7 Hz, 2H, ArH), 6.96 (d, ^4J = 2.0 Hz, 1H, ArH), 7.15 (d, ^3J = 8.7 Hz, 1H, ArH), 7.52 (d, ^3J = 8.5 Hz, 2H, ArH), 9.05 (s, 1H, OH), 9.92 (s, 1H, OH). MS (EI, 300 °C): *m*/*z* (%) = 443 $[M]^{+\cdot}$ (2.3), 332 (2.3), 226 (59.4), 113 (5.5), 98 (100.0). IR (film): ν = 3365 (m), 3117 (s), 2954 (s), 2872 (m), 1609 (s), 1512 (s), 1476 (m), 1461 (m), 1246 (s), 843 (m), 820 (m). Anal. Calcd for $C_{27}H_{29}N_3O_3$: C, 73.11; H, 6.59; N, 9.47. Found: C, 73.14; H, 6.61; N, 9.50.

5.2.4.5. 6-Hydroxy-2-(4-hydroxyphenyl)-1-[4-(2-piperidin-1-ylethoxy)benzyl]-1*H*-benzimidazole (7e). From **6c** (263 mg, 0.56 mmol). Yield: 124 mg (50%), colorless oil. ^{1}H NMR (DMSO-d_6): δ = 1.30–1.41 (m, 2H, CH_2), 1.41–1.51 (m, 4H, CH_2), 2.34–2.44 (m, 4H, CH_2), 2.57–2.65 (m, 2H, =N–CH_2–), 3.99 (t, ^3J = 5.8 Hz, 2H, –O–CH_2–), 5.32 (s, 2H, phenyl-CH_2–), 6.63 (d, ^4J = 1.9 Hz, 1H, ArH), 6.68 (dd, ^3J = 8.5 Hz, ^4J = 2.1 Hz, 1H, ArH), 6.86 (d, ^3J = 8.6 Hz, 2H, ArH), 6.87 (d, ^3J = 8.9 Hz, 2H, ArH), 6.93 (d, ^3J = 8.6 Hz, 2H, ArH), 7.42 (d, ^3J = 8.6 Hz, 1H, ArH), 7.48 (d, ^3J = 8.5 Hz, 2H, ArH), 9.21 (s, 1H, OH), 9.89 (s, 1H, OH). MS (EI,

300 °C): *m*/*z* (%) = 443 $[M]^{+\cdot}$ (2.3), 332 (2.3), 226 (59.4), 113 (5.5), 98 (100.0). IR (film): ν = 3168 (m), 2959 (m), 2875 (m), 1610 (s), 1512 (m), 1479 (m), 1462 (m), 1240 (s), 1217 (s), 845 (m), 819 (m). Anal. Calcd for $C_{27}H_{29}N_3O_3$: C, 73.11; H, 6.59; N, 9.47. Found: C, 73.32; H, 6.73; N, 9.42.

5.3. Structural characterization

The assignment of the 2-phenyl-1*H*-benzimidazoles **7b–e** to either the 5- or 6-OH derivatives was performed by nuclear Overhauser effect experiments (NOE). Saturation of the benzylic protons at 2167 Hz or 2133 Hz led to a saturation transfer to the *ortho*-standing protons at the C2-aryl as well as the *ortho*-protons at the benzyl ring. A pronounced NOE effect is observed to the C7-H. The splitting of the C7-H signal of **7b** into a doublet with ^3J = 8.7 Hz indicated an *ortho*-coupling as realized in 5-OH isomers, while the same NOE in experiments with **7c** results in a doublet with a *meta*-coupling of ^4J = 2.1 Hz (6-OH isomer). The same effects were determined for **7d** (5-OH) and **7e** (5-OH).

Two absorption maxima at 240–270 nm and 290–330 nm were detectable in the UV-spectra of the compounds **7a–e** (Table 1, Supplementary data). While the first is usually not well separated, the latter can be used for UV analysis. The λ_{max},abs of **7a** appeared in methanol at 291 nm. A 5-OH group shifted the maximum to 309 nm (**7b**, **7d**) and a 6-OH group to 304 nm (**7c**, **7e**). This effect is nearly identical in methanol, PBS and 0.1 N HCl. If 0.1 N NaOH is used, the deprotonation of the phenolic groups led to a bathochromic shift of about 20 nm.

The emission spectra are strongly dependent on the presence and the position of the hydroxyl groups at the benzimidazole core. Compound **7a** showed in methanol λ_{max},em at 351 nm which is shifted by a 5-OH group to 409 nm (**7b**) and 380 nm (**7d**) and by a 6-OH group to 390 nm (**7c**) and 374 nm (**7e**), respectively. Measurement of the compounds in aqueous solution (PBS, 0.1 N HCl, 0.1 N NaOH) led to fluorescence emissions at higher wavelength. In some cases, a second maximum appeared in the spectra.

This dual emission might be due to fluorescent impurities in solution. However, it is very unlikely since all of these compounds appeared to be homogeneous by HPLC analysis under the various conditions used to separate the isomers. Dual fluorescence emission is frequently observed when there is emission from two distinct excited-state species whose interconversion takes place at a rate comparable to their rates of fluorescence.[40]

5.4. Biological methods

5.4.1. In vitro assay on human ERα and ERβ recombinants

Ligand binding was evaluated in a semi-solid radiometric assay based on the propensity of ERα or ERβ to adsorb onto hydroxylapatite (HAP) at low ionic strength.[28] For that purpose, highly purified recombinant hERα (Calbiochem®) and hERβ (Calbiochem®), respectively, was adsorbed onto HAP (hERα and hERβ dilution in 10 mM Tris·HCl pH 8, final system concentration 10 nM). HAP was incubated 3 h at room temperature with $[^3H]E_2$ (5 nM, Perkin–Elmer) with or without increasing amounts of investigated compounds or unlabeled E_2 (Sigma). Radioactive material adsorbed onto HAP was then extracted with ethanol and measured by liquid scintillation counting. Relative concentrations of investigated compounds and E_2 required to reduce the binding of $[^3H]E_2$ by 50% gave their RBA value; RBA = ($[IC_{50}]E_2/[IC_{50}]$compound) × 100. Assays were performed twice, each time in duplicate.

5.4.2. Luciferase assay with ERα-positive MCF-7-2a cells stably transfected with the reporter plasmid ERE_{wtc}luc

The relevant in vitro assay was described earlier by Hafner et al.[29] At least 24 h before starting the experiment MCF-7-2a cells were cultivated in DMEM supplemented with dextran/charcoal-treated FCS (ct-FCS, 5%). Cells from an almost confluent monolayer were removed by trypsinization and suspended to approximately 2.2×10^5 cells/mL in the growth medium mentioned above. The cell suspension was then cultivated in 96-well plates (100 μL/well) at growing conditions (see above). After 24 h, 25 μL of a stock solution of the test compounds were added to achieve concentrations ranging from 10^{-5} to 10^{-11} M and the plates were incubated for 48 h. After 48 h the test medium was removed and cells were lysed (50 μL/well CCLR, 30 min, 600 rpm, rt). Luciferase activity was assayed using the Promega luciferase assay (50 μL/well). Luminescence (in relative light units, RLU) was measured for 10 s using a microlumat. Estrogenic activity was expressed as % activation of a 10^{-8} M E_2 control (100%).

To evaluate the antagonistic activity, the cells were incubated with the test compounds in concentrations from 10^{-5} to 10^{-11} M along with a constant amount of E_2 (10^{-9} M). The concentration of the compound, which is necessary to reduce the effect of E_2 by 50%, is IC_{50}.

5.4.3. Biological methods

5.4.3.1. Cell culture. The human MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, USA). Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay.[42] The MCF-7 cells were maintained in L-glutamine containing Eagle's MEM (Sigma, Germany), supplemented with $NaHCO_3$ (2.2 g/L), sodium pyruvate (110 mg/L), gentamycin (50 mg/L) and 10% fetal calf serum (FCS; Gibco, Germany) using 75 cm^2 culture flasks in a humidified atmosphere (95% air/5% CO_2) at 37 °C. The MDA-MB-231 cells (McCoýs 5A medium supplemented with $NaHCO_3$ (2.2 g/L), sodium pyruvate (110 mg/L), gentamycin (50 mg/L) and 5% FCS) were maintained under the same conditions. The cell lines were weekly passaged after treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (0.02%; EDTA; Boehringer, Germany). Mycoplasma contamination was regularly monitored and only mycoplasma-free cultures were used.

5.4.3.2. In vitro chemosensitivity assays. The in vitro testing of the 1*H*-benzimidazoles for antitumor activity was carried out on exponentially dividing human cancer cells according to a previously published microtiter assay.[30,31] Briefly, in 96-well microtiter plates 100 μL of a cell suspension at 7700 cells/mL culture medium (MCF-7) resp. at 3200 cells/mL (MDA-MB-231) were plated into each well and incubated at 37 °C for 3 days. By addition of an adequate volume of a stock solution of the respective compound (solvent: DMF) to the medium the aimed test concentration was obtained. Sixteen wells were used for each test concentration and for the control, which contained the corresponding amount of DMF. After the proper incubation time the medium was removed, the cells were fixed with a glutardialdehyde solution and stored under phosphate buffered saline (PBS) at 4 °C. Cell biomass was determined by a crystal violet staining technique as described earlier.[30,31] The efficiency of the complexes is expressed as corrected % T/C_{corr} values according to the following equations:

$$\text{Cytostatic effect}: T/C_{corr}[\%] = [(T - C_o)/(C - C_o)] \times 100$$

where T (test) and C (control) were the optical densities at 590 nm of the crystal violet extract of the cells in the wells (i.e., the chromatin-bound crystal violet extracted with ethanol 70%), and C_o was the density of the cell extract immediately before treatment.

$$\text{Cytocidal effect}: \tau[\%] = [(T - C_o)/C_o] \times 100.$$

A microplate reader (Flashscan S12 AnalytikJena AG) was used for the automatic estimation of the optical density of the crystal violet extract in the wells.

5.4.4. Thermal denaturation temperatures of DNA

The thermal denaturation temperatures T_m were determined according to an already published procedure.[43]

5.4.5. Determination of absorption and emission spectra

The UV-spectra were measured at 225–400 nm using 96-well UV-plates. Methanol, PBS (pH 7.3), 0.1 M HCl and 0.1 M NaOH were used as solvent for the dilution of a 1×10^{-2} M stock solution to a final concentration of 1×10^{-4} M. The absorption of a 200 μL/well proportion of each solution was quantified with a microplate reader (Flashscan S12 AnalytikJena).

Fluorescence spectra were analogously recorded with a Fluorimeter F4500 (Hitachi) with a scan speed of 240 nm/min (2D) and 1200 nm/min (3D), respectively, and an excitation and emission slot of 5.0 nm. DNA and HSA interactions were determined in 0.1% DNA (1 mg/mL) and HSA (1 mg/mL) solutions using a final drug concentration 1 μM.

5.4.6. Cellular uptake studies

In 6-well microtiter plates 2 mL of a cell suspension at 8×10^4 MCF-7 cells/mL culture medium were plated into each well and incubated at growing conditions. Test compounds were dissolved to an 1 mM stock solution in methanol, which was further diluted with FCS free medium to a final concentration of 10 μM. The medium was removed after 5 days and replaced by 2 mL of drug-containing medium or medium containing 0.1% (v/v) of the corresponding solvent as negative control. After the respective incubation periods, the medium was removed; the cells were washed with 1 mL of PBS, trypsinated and suspended in medium. Cell pellets, obtained by centrifugation were stored at 4 °C. For the measurement, the pellets were respectively re-suspended in 400 μL of an 0.1% aqueous sodium dodecylsulfate solution and sonificated. An amount of 300 μL of the cell suspension was diluted with 100 μL of methanol and measured in a fluorimeter with an excitation wavelength of 300 nm (λ_{max}em = 490 nm).

For protein determination,[41] 200 μL of Bradford reagent (diluted 1:5 directly before the experiment) were transferred in 96-wells microtiter plates and 20 μL of cell suspension were added and mixed carefully. After 20 min of incubation on a rotary shaker, absorption was measured at 595 nm in a microplate reader (Flashscan S12 AnalytikJena AG). An aqueous solution of humane serum albumin (HSA) was used for calibration. Samples were performed in duplicate. Results were expressed as means of 6-wells as pmol drug per μg protein.

For fluorescence measurements six cell pellets were used for generation of a calibration curve (0, 75, 150, 300, 500 and 750 nM). The fluorescence values were related to the protein concentration.

Using the cellular protein-volume relation[44] of 8.85 pL/ng the intracellular concentration was calculated:

$$c[\mu M] = (\mu mol/\mu g \times 1000)/(8.85\ pL/ng).$$

5.4.7. Adenosine receptor binding assays

Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the assays being 2.5%. The radio-ligands and their concentrations were as follows: [^{3}H]CCPA, 0.5 nM (A_1), [^{3}H]MSX-2, 1 nM (A_{2A}), and [^{3}H]PSB-11, 0.5 nM (A_3). Binding assays were performed essentially as described.[45–47] Rat brain cortical membranes were used as a source of A_1 receptors, rat brain striatal membranes for A_{2A} receptors, and membranes from Chinese hamster ovary (CHO) cells recombinantly expressing the human A_3 receptor for adenosine A_3 receptor assays. Membranes (ca. 70 μg protein) were preincubated for 20 min with 0.12–0.22 IU/mL of adenosine deaminase in order to remove endogenous adenosine. Compounds were initially screened at a final concentration of 10 μM. If the inhibition of radio-ligand binding was >50%, curves were determined using 6–7 different concentrations of test compounds spanning three orders of magnitude. At least three separate experiments were performed, each in triplicate. For nonlinear regression analysis, the Cheng-Prusoff equation and KD values of 0.2 nM (rat A_1) for [^{3}H]CCPA, 8 nM (rat A_{2A}) for [^{3}H]MSX-2, and 4.9 nM (human A_3) for [^{3}H]PSB-11 were used to calculate K_i values from IC_{50} values.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.016.

References and notes

1. Schaudig, K.; Schwenkhagen, A. *Gynaekol. Endokrinol.* **2008**, *6*, 205.
2. Oseni, T.; Patel, R.; Pyle, J.; Jordan, V. C. *Planta Med.* **2008**, *74*, 1656.
3. Hosoi, T. *Clin. Calcium* **2007**, *17*, 1419.
4. Jordan, V. C.; O'Malley, B. W. *J. Clin. Oncol.* **2007**, *25*, 5815.
5. Leung, F. P.; Tsang, S. Y.; Wong, C. M.; Yung, L. M.; Chan, Y. C.; Leung, H. S.; Yao, X.; Huang, Y. *Clin. Exp. Pharmacol. Physiol.* **2007**, *34*, 809.
6. Jordan, V. C. *Eur. J. Cancer* **2008**, *44*, 30.
7. Lewis-Wambi, J. S.; Jordan, V. C. *Breast Dis.* **2006**, *24*, 93.
8. Musa, M. A.; Khan, M. O. F.; Cooperwood, J. S. *Curr. Med. Chem.* **2007**, *14*, 1249.
9. Robertson, J. F. R. *Cancer Treat. Rev.* **2004**, *30*, 695.
10. Hajela, K.; Jha, A. K.; Pandey, J. *Curr. Med. Chem.* **2001**, *1*, 235.
11. Abu Fanne, R.; Brzezinski, A.; Danenberg, H. D. *Mini-Rev. Med. Chem.* **2007**, *7*, 871.
12. Hansdottir, H. *Clin. Interv. Aging* **2008**, *3*, 45.
13. Gennari, L.; Merlotti, D.; Valleggi, F.; Martini, G.; Nuti, R. *Drugs Aging* **2007**, *24*, 361.
14. Biberger, C.; von Angerer, E. *J. Steroid Biochem. Mol. Biol.* **1998**, *64*, 277.
15. von Angerer, E.; Strohmeier, J. *J. Med. Chem.* **1987**, *30*, 131.
16. Gust, R.; Keilitz, R.; Schmidt, K. *J. Med. Chem.* **2001**, *44*, 1963.
17. Gust, R.; Keilitz, R.; Schmidt, K.; von Rauch, M. *J. Med. Chem.* **2002**, *45*, 3356.
18. Gust, R.; Keilitz, R.; Schmidt, K. *J. Med. Chem.* **2002**, *45*, 2325.
19. Gust, R.; Keilitz, R.; Schmidt, K.; von Rauch, M. *Arch. Pharm.—Pharm. Med. Chem.* **2002**, *335*, 463.
20. Gust, R.; Busch, S.; Keilitz, R.; Schmidt, K.; von Rauch, M. *Arch. Pharm.—Pharm. Med. Chem.* **2003**, *336*, 456.
21. Wiglenda, T.; Ott, I.; Kircher, B.; Schumacher, P.; Schuster, D.; Langer, T.; Gust, R. *J. Med. Chem.* **2005**, *48*, 6516.
22. Wiglenda, T.; Gust, R. *J. Med. Chem.* **2007**, *50*, 1475.
23. Hayashi, S. I.; Eguchi, H.; Tanimoto, K.; Yoshida, T.; Omoto, Y.; Inoue, A.; Yoshida, N.; Yamaguchi, Y. *Endocr. Relat. Cancer* **2003**, *10*, 193.
24. Brzozowski, A. M.; Pike, A. C. W.; Dauter, Z.; Hubbard, R. E.; Bonn, T.; Engström, O.; Öhman, L.; Greene, G. L.; Gustafsson, J.-Å.; Carlquist, M. *Nature* **1997**, *389*, 753.
25. Becker, H.; Berger, W.; Domschke, G.; Fanghänel, E.; Faust, J.; Fischer, M.; Gentz, F.; Gewald, K.; Gluch, R.; Mayer, R.; Müller, K.; Pavel, D.; Schmidt, H.; Schollberg, K.; Schwetlick, K.; Seiler, E.; Zeppenfeld, G. *Organikum, Johann Ambrosius Barth Leipzig, Kapitel D.5*, 15, Auflage, 1977.
26. Dauwe, C.; Buddrus, J. *Synthesis* **1995**, 171.
27. Grese, T. A.; Cho, S.; Finley, D. R.; Godfrey, A. G.; Jones, C. D.; Lugar, C. W., III; Martin, M. J.; Matsumoto, K.; Pennington, L. D.; Winter, M. A.; Adrian, M. D.; Cole, H. W.; Magee, D. E.; Phillips, D. L.; Rowley, E. R.; Short, L. L.; Glasebrook, A. L.; Bryant, H. U. *J. Med. Chem.* **1997**, *40*, 146.
28. Maaroufi, Y.; Leclercq, G. *J. Steroid Biochem. Mol. Biol.* **1994**, *48*, 155.
29. Hafner, F.; Holler, E.; von Angerer, E. *J. Steroid Biochem. Mol. Biol.* **1996**, *58*, 385.
30. Bernhardt, G.; Reile, H.; Birnböck, H.; Spruß, T.; Schönenberger, H. *J. Cancer Res. Clin. Oncol.* **1992**, *118*, 35.
31. Reile, H.; Birnböck, H.; Bernhardt, G.; Spruß, T.; Schönenberger, H. *Anal. Biochem.* **1990**, *187*, 262.
32. Gust, R.; Lubczyk, V. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 75.
33. Colquhoun, A.; Newsholme, E. A. *Cell Biochem. Funct.* **1997**, *15*, 135.
34. Catherine, P.; Barry, E. *Cancer Res.* **2000**, *60*, 1887.
35. Kubota, Y.; Nakamura, H. *Chem. Lett.* **1991**, 745.
36. Hasegawa, N.; Kubota, Y. *Nucleic Acids Symp. Ser.* **1993**, *29*, 85.
37. Suh, D.; Chaires, J. B. *Bioorg. Med. Chem.* **1995**, *3*, 723.
38. Grese, T. A.; Sluka, J. P.; Bryant, H. U.; Cullinan, G. J.; Glasebrook, A. L.; Jones, C. D.; Matsumoto, K.; Palkowitz, A. D.; Sato, M.; Termine, J. D.; Winter, M. A.; Yang, N. N.; Dodge, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14105.
39. Sorbera, L. A.; Castañer, J.; Silvestre, J. S. *Drugs Future* **2002**, *27*, 942.

40. Ireland, J. F.; Wyatt, P. A. H. *Adv. Phys. Org. Chem.* **1976**, *12*, 131.
41. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
42. Hay, R. J. *Anal. Biochem.* **1988**, *171*, 225.
43. Hille, A.; Ott, I.; Kitanovic, A.; Kitanovic, I.; Alborzinia, H.; Lederer, E.; Wölfl, S.; Metzler-Nolte, N.; Schäfer, S.; Sheldrick, W.; Bischof, C.; Schatzschneider, U.; Gust, R. *J. Biol. Inorg. Chem.* **2009**, *14*, 711.
44. Gust, R.; Schnurr, B.; Krauser, R.; Bernhardt, G.; Koch, M.; Schmid, B.; Hummel, E.; Schönenberger, H. *J. Cancer Res. Clin. Oncol.* **1998**, *124*, 585.
45. Müller, C. E.; Maurinsh, J.; Sauer, R. *Eur. J. Pharm. Sci.* **2000**, *10*, 259.
46. Yan, L.; Müller, C. E. *J. Med. Chem.* **2004**, *47*, 1031.
47. Müller, C. E.; Diekmann, M.; Thorand, M.; Ozola, V. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 501.