Supporting Information

Synthesis and Pharmacological Evaluation of *1H*-Imidazoles as Ligands for the Estrogen Receptor and Cytotoxic Inhibitors of the Cyclooxygenase

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Content

Experimental Section

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General Methods. All reagents and solvents were purchased from ACROS ORGANICS, Fluka Chemie, Lancaster, Merck or Sigma-Aldrich. Reactions were all monitored by TLC, performed on silica gel plates 60 F_{254} (Merck, Darmstadt/Germany). Visualization on TLC was achieved by UV light. Column chromatography was performed with Merck silica gel 60H, grain size < 0.063 mm, 230 mesh ASTM (Darmstadt/Germany). Melting points: 510 Büchi (Flawil/Schweiz) capillary melting point apparatus. IR spectra (KBr pellets): Perkin Elmer Model 580 A (Rodgau-Jügesheim/Germany). ^{1}H NMR: Avance DPX-400 spectrometer (Bruker, Karlsruhe/Germany) at 400 MHz, AMX 500 spectrometer (Bruker, Karlsruhe/Germany) at 500.14 MHz, (internal standard: TMS), Elemental analyses: Microlaboratory of the Free University of Berlin. EI-MS spectra: CH-7A-Varian MAT, 70 eV (Melbourne/Australia). FAB spectra: CH5 DF (Varian MAT, Bremen, Germany) modified with focused FAB-gun (AMD-Intectra). Microlumat: VICTOR^2, 1420 Multilabel Counter (Wallac, Perkin Elmer, Life sciences, Turku/Finnland). Microplate reader: FLASHscan S12 (analytikjena AG/Germany).

Synthesis

General Procedure for the Preparation of *N*-Arylbenzamidine
A solution of anisidine in dry toluene (20 – 40 mL) was treated with $NaNH_2$ under N_2 atmosphere. Benzonitrile was then added after being stirred for 3 h at 130 – 140 °C (oil bath). The mixture was warmed to 120 – 140 °C for 3 h and cooled overnight to room temperature without stirring.

***N*-(4-Methoxyphenyl)-4-methoxybenzamidine (3a)**
From anisidine (2.43 g, 19.76 mmol), $NaNH_2$ (924 mg, 23.70 mmol) and benzonitrile **1** (2.63 g, 19.75 mmol). The crude product **3a** was separated by suction filtration and washed with toluene, suspended in H_2O (150 mL) and subsequently stirred for 30 min. The slurry was extracted with CH_2Cl_2 (3 x 60 mL), the organic layers were combined and dried over Na_2SO_4. Evaporation gave the product **3a** (2.70 g, 53 % yield) as an amorphous white solid (mp: 150 °C). TLC Si_2O (ethanol): R_f = 0.5. ^{1}H NMR ([D_6]DMSO): δ = 7.92 (d, 2H, J = 8.8 Hz, Ar*H*), 6.96 (d, 2H, J = 8.8 Hz, Ar*H*), 6.88 (d, 2H, J = 8.8 Hz, Ar*H*), 6.76 (d, 2H, J = 8.7 Hz, Ar*H*), 6.07 (s, 2H, NH_2), 3.79 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3). MS (EI, 80 °C): m/z (%) = 255 $[M]^{+\bullet}$ (27), 134 (33), 133 (100), 108 (25), 90 (29), 44 (27). IR (KBr): $\bar{\nu}$ = 3473 (w), 3349 (w), 2950 (w), 2834 (w), 1616 (s), 1515 (s), 1380 (m), 1255 (s), 1231 (s), 1173 (m), 1030 (s), 837 (s).

***N*-(4-Methoxyphenyl)-2-chloro-4-methoxybenzamidine (3b)**
From anisidine (2.59 g, 21.07 mmol), $NaNH_2$ (986 mg, 25.27 mmol) and benzonitrile **2** (3.53 g, 21.06 mmol). Water (150 mL) was added to the crude mixture and extracted repeatedly with CH_2Cl_2 (3 x 50 mL). The organic layers were combined, dried over Na_2SO_4 and concentrated in vacuum. The crude product was purified by chromatography on silica gel (CH_2Cl_2/methanol: 95/5) to give **3b** (1.08 g, 18 % yield) as vitreous violet solid (mp: 79 °C). TLC SiO_2 (ethanol): R_f = 0.6. ^{1}H NMR ([D_6]DMSO): δ = 9.50 (ws, 2H, NH_2), 7.72 (bs, 1H, Ar*H*), 7.28 (ws, 3H, Ar*H*), 7.09 (ws, 3H, Ar*H*), 3.85 (ws, 3H, OCH_3), 3.73 (ws, 3H, OCH_3). MS (EI, 180 °C): m/z (%) = 290 $[M]^{+\bullet}$ (71), 275 (26), 168 (52), 123 (100), 108 (90). IR (KBr): $\bar{\nu}$ = 3432 (m), 3284 (m), 3086 (m), 1636 (s), 1610 (s), 1504 (s), 1383 (m), 1292 (m), 1240 (s), 1221 (s), 1033 (s), 858 (m), 828 (m), 814 (m), 745 (w).

General Procedure for the Preparation of 1,2,4-Triaryl-*1H*-imidazoles.
K_2CO_3 and α-bromoketone were added subsequently to a stirred solution of the *N*-arylbenzamidine in $CHCl_3$ (3.00 mL) and H_2O (0.50 mL) at room temperature. The reaction was kept at room temperature for 18 – 56 h, was then quenched with H_2O (50 mL) and stirred for additional 20 min. The organic layer was separated, dried over Na_2SO_4 and evaporated under reduced pressure, to give a crude product which was purified by chromatography on silica gel.

1,2,4-Tris(4-methoxyphenyl)-*1H*-imidazole (6a).
From amidine **3a** (450 mg, 1.76 mmol), α-bromoketone **5a** (528 mg, 2.30 mmol) and K_2CO_3 (320 mg, 2.32 mmol); reaction time 20 h. Yield: 430 mg (63 %); colourless solid (mp: 131 °C). TLC SiO_2

(Et_2O): R_f = 0.9. ^{1}H NMR ([D_6]DMSO): δ = 7.79 (d, 2H, J = 8.8 Hz, Ar*H*), 7.74 (s, 1H, 5-*H*), 7.31 (d, 2H, J = 8.9 Hz, Ar*H*), 7.28 (d, 2H, J = 8.9 Hz, Ar*H*), 7.03 (d, 2H, J = 8.9 Hz, Ar*H*), 6.96 (d, 2H, J = 8.9 Hz, Ar*H*), 6.88 (d, 2H, J = 8.9 Hz, Ar*H*), 3.80 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3). MS (EI, 150 °C): m/z (%) = 386 $[M]^{+\bullet}$ (100), 253 (35), 238 (35). IR (KBr): $\bar{\nu}$ = 3433 (w), 2934 (w), 2834 (w), 1611 (m), 1513 (s), 1498 (s), 1463 (m), 1299 (m), 1248 (s), 1172 (m), 1030 (m), 834 (m).

4-(2-Chloro-4-methoxyphenyl)-1,2-bis(4-methoxyphenyl)-*1H*-imidazole (6b)
From amidine **3a** (232 mg, 0.91 mmol), α-bromoketone **4** (200 mg, 0.76 mmol) and K_2CO_3 (166 mg, 1.20 mmol); reaction time 21 h; column chromatography with petrol ether/diethyl ether: 3/1. Yield: 185 mg (48 %); colourless solid (mp: 134 °C). TLC SiO_2 (petrol ether/diethyl ether 3:1): R_f = 0.1. ^{1}H NMR ([D_6]DMSO): δ = 8.20 (d, 1H, J = 8.3 Hz, Ar*H*), 7.70 (s, 1H, 5-*H*), 7.32 (d, 2H, J = 8.9 Hz, Ar*H*), 7.30 (d, 2H, J = 9.0 Hz, Ar*H*), 7.15 (d, 1H, J = 2.0 Hz, Ar*H*), 7.09 (dd, 1H, J = 2.0, J = 8.4 Hz, Ar*H*), 7.04 (d, 2H, J = 8.9 Hz, Ar*H*), 6.89 (d, 2H, J = 8.8 Hz, Ar*H*), 3.93 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3). MS (EI, 260 °C): m/z (%) = 435 $[M]^{+\bullet}$ (100), 134 (43). IR (KBr): $\bar{\nu}$ = 3433 (w), 2936 (w), 1611 (w), 1513 (s), 1486 (m), 12150 (s), 1297 (w), 1178 (w), 1030 (m), 835 (m).

2,4-Bis(2-chloro-4-methoxyphenyl)-1-(4-methoxyphenyl)-*1H*-imidazole (6c)
From amidine **3b** (262 mg, 0.90 mmol), α-bromoketone **4** (200 mg, 0.76 mmol) and K_2CO_3 (111 mg, 0.80 mmol); reaction time: 18 h; column chromatography with gradual elution: petrol ether/diethyl ether: 1/1, 1/2, 1/4, diethyl ether. Yield: 328 mg (80 %); colourless solid (mp: 136 °C). TLC SiO_2 (petrol ether/diethyl ether 2:1): R_f = 0.4. ^{1}H NMR ([D_6]DMSO): δ = 8.11 (d, 1H, J = 8.4 Hz, Ar*H*), 7.83 (s, 1H, 5-*H*), 7.49 (d, 1H, J = 8.5 Hz, Ar*H*), 7.20 – 7.16 (m, 3H, Ar*H*), 7.06 (dd, 1H, J = 2.0 Hz, J = 8.3 Hz, Ar*H*), 7.03 (d, 1H, J = 2.5 Hz, Ar*H*), 6.97 (dd, 1H, J = 2.5 Hz, J = 8.6 Hz, Ar*H*), 6.93 (d, 2H, J = 8.9 Hz, Ar*H*), 3.95 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3). MS (EI, 220 °C): m/z (%) = 454 $[M]^{+\bullet}$ (90) 453 (58), 287 (28), 274 (100), 120 (32), 92 (28), 77 (33). IR (KBr): $\bar{\nu}$ = 3434 (w), 2961 (w), 2936 (w), 1608 (m), 1514 (s), 1484 (m), 1463 (m), 1293 (m), 1249 (s), 1200 (m), 1043 (m), 1028 (m), 836 (m).

5-Ethyl-1,2,4-tris(4-methoxyphenyl)-*1H*-imidazole (6d)
From amidine **3a** (282 mg, 1.10 mmol), α-bromoketone **5b** (250 mg, 0.97 mmol) and K_2CO_3 (202 mg, 1.46 mmol); reaction time: 48 h; column chromatography with CH_2Cl_2/methanol: 98/2. Yield: 99 mg (22 %); colourless solid (mp: 135 °C). TLC Si_2O (CH_2Cl_2/methanol: 9/1): R_f = 0.9. ^{1}H NMR ([D_6]DMSO): δ = 7.65 (d, 2H, J = 8.7 Hz, Ar*H*), 7.32 (d, 2H, J = 8.8 Hz, Ar*H*), 7.26 (d, 2H, J = 8.9 Hz, Ar*H*), 7.07 (d, 2H, J = 8.8 Hz, Ar*H*), 7.00 (d, 2H, J = 8.8 Hz, Ar*H*), 6.82 (d, 2H, J = 8.8 Hz, Ar*H*) , 3.83 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 2.58 (q, 2H, J = 7.3Hz, CH_2CH$_3$), 0.94 (t, 3H, J = 7.4 Hz, CH$_2$CH_3). MS (EI, 140 °C): m/z (%) = 414 $[M]^{+\bullet}$ (44), 399 (24), 162 (26), 123 (48), 120 (100), 107 (34), 57 (32). IR (KBr): $\bar{\nu}$ = 3483 (w), 2963 (w), 2934 (w), 1610 (w), 1509 (s), 1463 (w), 1297 (w), 1248 (s), 1177 (w), 1104 (w), 1029 (m), 837 (m).

1,2-Bis(4-methoxyphenyl)-4-(trifluoromethyl)-*1H*-imidazole (6e)
From amidine **3a** (300 mg, 1.17 mmol), K_2CO_3 (540 mg, 3.91 mmol) and 1-bromo-3-trifluoroacetone **5c** (3 x 100 µL, 2.86 mmol, after 15 h and 32 h). After the last addition, the reaction mixture was stirred for 24 h. The crude material was purified by column chromatography with gradual elution: CH_2Cl_2/methanol, 98:2, 95:5, 9:1. Yield: 158 mg (39 %); colourless solid (mp: 68 °C). TLC Si_2O (CH_2Cl_2/methanol 9:1): R_f = 0.6. ^{1}H NMR ([D_6]DMSO): δ = 7.40 (d, 2H, J = 8.8 Hz, Ar*H*), 7.10 (s, 1H, 5-*H*), 6.95 (d, 2H, J = 8.6 Hz, Ar*H*), 6.90 (d, 2H, J = 8.8 Hz, Ar*H*), 6.86 (d, 2H, J = 8.9 Hz, Ar*H*), 3.75 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3). MS (EI, 100°C): m/z (%) = 365 (25), 348 $[M]^{+\bullet}$ (2), 233 (45), 136 (100), 134 (31). FAB(+) (matrix: DMSO/m-NO_2-benzyl-OH): 367 (100), 349 $[M + H]^+$ (10). IR (KBr): $\bar{\nu}$ = 3395 (w), 3104 (w), 2840 (w), 1611 (s), 1512 (s), 1280 (m) 1252 (s), 1181 (s), 1031 (m), 838 (m).

Molecular Modelling

The pharmacophore model description refers to Rollinger et al.. [26] The software package CATALYST [27] was used for molecular modelling (3D molecular structure generation, conformational analysis, and

pharmacophore fitting calculation). As outlined in reference [26], the pharmacophore model COX-1-A was manually built based upon the atomic coordinates published in the PDB entry 1CQE, representing the complex between COX-1 and the inhibitor flurbiprofen. For COX-2, two different pharmacophore modeling approaches had been used: i) a structure-based model (COX-2-A) was manually generated starting from the atomic coordinates published in the PDB entry 1CX2, representing the COX-2/SC-558 complex, and ii) an automatically built ligand-based pharmacophore model (COX-2-B, starting from four different COX-2 inhibitors: SC-558, celecoxib, rofecoxib, and nimesulide. The structure-based model was used in this study.

Compounds **7a-e** were constructed within Catalyst and 3D structures were generated using the standard 2D-3D conversion algorithm. Conformational analysis was performed using the BEST algorithm (maximum set of 250 conformations, retaining all conformers within a 20 kcal/mol energy threshold above the calculated global potential energy minimum). The pharmacophore fitting operation was performed using the FAST FLEXIBLE option. The observed occupation of features was compared with ligand binding information from the initial crystal structures.

Biological Methods

Transcriptional binding assay

The transactivation on the estrogen receptor was tested using MCF-7 2a cells, stably transfected with the plasmid ERE_{wtc}luc. One week before starting the experiment, the cells were cultivated in DMEM supplemented with L-glutamine, antibiotics and dextran/charcoal-treated BCS (ct-BCS, 50 mL/L). Cells from an almost confluent monolayer were removed by trypsinization and suspended to approximately 2.2 x 10^5 cells/mL in the growth medium mentioned above. The cell suspension was then cultivated in six well flat-bottomed plates (0.5 mL cell suspension and 1.5 mL medium per well) at growing conditions (see above). After 24 h, 20 µL of a stock solution of the test compounds were added to achieve concentrations ranging from 10^{-5} - 10^{-10} M and the plates were incubated for 50 h. Before harvesting, the cells were washed twice with PBS and then 200 µL of cell culture lysis reagent were added into each well. After a 20 min lysation at room temperature cells were transferred into reaction tubes and centrifuged. Luciferase was assayed using the Promega luciferase assay reagent. 50 µL of each supernatant were mixed with 50 µL of substrate reagent. Luminescence (in relative light units, RLU) was measured for 10 s using a microlumat. Measurements were corrected by correlating the quantity of protein (quantified according to Bradford [28]) of each sample with the activity of luciferase. Estrogenic activity was expressed as % activation of a 10^{-8} M estradiol control (100 %).

In vitro chemosensitivity assay

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. [29] 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 (McCoy´s 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.

In vitro chemosensitivity assays

The *in vitro* testing of the *1H*-imidazoles for antitumor activity was carried out on exponentially dividing human cancer cells. 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. For each test

concentration and for the control, which contained the corresponding amount of DMF, 16 wells were used. After the appropriate 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. [18,19] The efficiency of the complexes is expressed as corrected % T/C_{corr} values according to the following equations:

Cytostatic effect: T/C_{corr} [%] = [(T-C_o) / (C-C_o)] x 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.

Cytocidal effect: τ [%] = [(T-C_o) / C_o] x 100.

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

COX inhibitor screening assay

A COX inhibition assay (COX Inhibitor Screening Assay) was performed as recommended by the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). In brief, ovine COX-1 and human recombinant COX-2 were incubated at 37 °C with heme and the compounds in final concentrations of 1, 10 and 200 µM for 10 min in a reaction buffer in an overall volume of 990 µL. The reaction was initiated by adding 10 µL of arachidonic acid. The test tube was vortexed and incubated for another 2 min at 37 °C. Enzyme catalysis was stopped by adding 50 µL of 1 M HCl. Test tubes were removed from the water bath and 100 µL of saturated stannous chloride solution was added. After 5 min at room temperature the stabile $PGF_{2\alpha}$ intermediate was afforded. The prostanoid product was quantified via EIA using a broadly specific antibody that binds to all the major prostaglandin compounds. A molecule of the analyte covalently attached to acetylcholine esterase (AchE) serves as the tracer. Quantification of the tracer was achieved by measuring its AchE activity with Ellman's reagent. This reagent consists of acetylthiocholin and 5,5'-dithio-bis(2-nitrobenzoic acid). The product of the AchE hydrolysis thiocholine reacts with the benzoic acid to provide 5-thio-2-nitrobenzoic acid which was measured at 412 nm.

ssDNA apoptosis ELISA

MDA-MB 231 cells cultivation was carried out as describe above. The amount of ssDNA (ssDNA Apoptosis ELISA Kit) was determined according to the manufacturer's protocol (Chemicon International, Temecula, CA, USA). MDA-MB 231 cells were seeded in triplicates at a concentration of 10000 cells/well in culture medium and incubated with the compounds at final concentrations of 1, 5, 10, 25 and 50 µM for 5 days at 37 °C in an overall volume of 150 µL. The cells were fixed with 80 % methanol in PBS on the 96-well plate, and the DNA denaturized with formamide at 75 °C. The DNA was quantified via ELISA using an antibody mixture that contains primary mAb to ssDNA and HRP-labeled anti-mouse secondary mAb. The wells were washed three times with buffer and an ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) solution was added. After 15-60 min the reaction was stopped by adding an HCl solution, and absorbance was read in a microplate reader at 405 nm. The ssDNA measurements were corrected by the cell concentration determined by the cell proliferation assay, EZ4U (Biomedica, Wien, Austria). The non-treated cell culture was set as 1.

Literature

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Table 1: Elemental analyses of target compounds **7a - 7e**.

Compound	Calculated (%)	Found (%)
7a	C 73.24 H 4.68 N 8.13	C 73.09 H 4.57 N 7.97
7b	C 66.58 H 3.99 N 7.40	C 66.72 H 4.25 N 7.16
7c	C 61.03 H 3.41 N 6.78	C 60.82 H 3.51 N 6.71
7d	C 74.18 H 5.41 N 7.52	C 74.17 H 5.77 N 7.31
7e	C 60.00 H 3.46 N 8.75	C 60.34 H 3.80 N 8.88