Oxidized analogs of Di(1H-indol-3-yl)methyl-4-substituted benzenes are NR4A1-dependent UPR inducers with potent and safe anti-cancer activity

SUPPLEMENTARY MATERIALS

Supplementary Table 1: Monitoring oxidation using HPLC

| Reaction time (h) | DIM-Ph-4-CF<sub>3</sub> | DIM-Ph-4-CF<sub>3</sub>OMs<sup>-</sup> | DIM-Ph-4-CO<sub>2</sub>Me | DIM-Ph-4-CO<sub>2</sub>MeOMs<sup>-</sup> |
|-------------------|-------------------------|---------------------------------|------------------------|---------------------------------|
| 0                 | 98                      | 0                               | 96                     | 0.9                             |
| 21                | 49                      | 39                              | 51                     | 34                              |
| 46                | 34                      | 39                              | 39                     | 43                              |

*a* Determined by HPLC using the same conditions as those for compound purity analyses (see below). After 46 h, the reactions were worked up, and DIM-Ph-4-XOMs<sup>-</sup>s were isolated in 36% and 32% yields, respectively.

Supplementary Table 2: HPLC analytical data for analogues using the A/B solvent system

| DIM-Ph-4-XOMs<sup>-</sup> | HPLC analysis |
|--------------------------|---------------|
|                          | t<sub>R</sub> (min) | peak area (%) |
| CF<sub>3</sub>           | 14.98         | 100           |
| CO<sub>2</sub>Me         | 13.70         | 98            |
| Cl                       | 15.08         | 96            |
| OMe                      | 14.07         | 100           |
| CO<sub>2</sub>H          | 12.66         | 96            |
| I(Ph-4-X)MI             |               |               |
| CF<sub>3</sub>           | 14.91         | 100           |
| CO<sub>2</sub>Me         | 13.74         | 100           |

Supplementary Table 3: Characteristics of leukemia cell lines --KG-1, MOLM-13, OCI-AML-2, OCI-AML-3 and THP-1 AML, K562 CML and MOLT-4 T-ALL<sup>**</sup>. See Supplementary Table_3
Supplementary Figure 1: Synthesis of DIM-Ph-4-Xs and structures of apoptosis-inducing agents. (A) Reagents and conditions: (a) CeCl$_3$$\cdot$7H$_2$O/NaI on silica gel, CH$_3$CN. (b) 5 M aq NaOH, MeOH, reflux; H$_2$O$^-$. (B) Chemical structures of NR4A1 agonist and apoptosis-inducer cytosporone B, NR4A1 antagonist ethyl 2-[6-(1-octanoyl)-2,3,4-trimethoxyphenyl]acetate (transcriptionally inactive), 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN/CD437), (E)-3-[[2-([1-adamantyl]-4-hydroxyphenyl]-1,3-pyrimid-5-yl]-2-propenoic acid (AHP3) and N-(4-hydroxyphenyl) all-trans-retinamide.
Supplementary Figure 2: Effects of DIM-Ph-4-Xs and the mesylate salts DIM-Ph-4-X+OMs on viability of cancer cell lines. Cancer cell lines having various metastatic potentials—HCT-116 colon, MCF-7 and MDA-MB-231 breast, and LNCaP, 22RV1, PC-3 and LAPC-4 prostate cancer—as measured by the MTT cell viability assay as described in the Methods. Cells were plated in 96-well plates, allowed to attach for 24 h and then treated with each compound at increasing concentrations (0.125 to 2.0 mM) in DMSO or DMSO alone (0.2% final concentration) for 72 h. Viability of treated cells relative to the vehicle-treated control is expressed as means of quadruplicates ± SD of the ratios of treated to control cells × 100. Concentration–viability response curves are represented as dashed lines and open symbols for the DIM-Ph-4-Xs (parent compounds) and as solid lines and closed symbols for the DIM-Ph-4-X+OMs (oxidized compounds). Individual compounds are designated by their X substituent at the phenyl ring 4-position (X = CF3, CO2Me, Cl, OMe and CO2H). IC50 values listed in Tables 1 and 2 were determined from these concentration–viability response curves.
Supplementary Figure 3: Effects of DIM-Ph-4-CF<sub>3</sub> and DIM-Ph-4-CF<sub>3</sub>+OMs<sup>-</sup> on leukemia cell viability as measured by changes in ATP levels. Cells were grown for 24 h in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin at 37° C and then treated for 24 h with compounds at the indicated concentrations in DMSO (0.125% final concentration) or DMSO alone. Data points represent the means of triplicates ± SD of the ratios of the viabilities of the treated to vehicle control cells × 100. Concentration–viability response curves for DIM-Ph-4-CF<sub>3</sub> and DIM-Ph-4-CF<sub>3</sub>+OMs<sup>-</sup> on (A) OCI-AML-2 (▲), MOLM-13 (◆), KG-1 (■), THP-1 (D) and OCI-AML-3 (●) AML cell lines and on (B) MOLT-4 T-ALL (+) and K562 CML (×) cell lines are represented by dashed lines and solid lines, respectively. IC<sub>50</sub> values were determined by interpolation of the concentration–viability curves and are listed in Table 3.

Supplementary Figure 4: Fetal bovine serum (10%) in growth medium does not have a major impact on AML growth inhibition by DIM-Ph-4-CF<sub>3</sub>+OMs<sup>-</sup>. MOLM-13 and OCI-AML-2 AML cells were grown in RPMI 1640 medium with and without 10% fetal bovine serum (FBS) and then treated for 24 h with 0.01, 0.1, 1.0 and 10.0 μM in DMSO (0.125% final concentration) or DMSO alone. Inhibition of cell viability was determined by measuring the decline in ATP levels as described in the Methods. Viabilities of treated cells relative to those of the vehicle-treated control cells were expressed as the ratios of means of triplicates ± SD × 100. Solid lines represent OCI-AML-2 (AML-2) (▲) and MOLM-13 (◆) cells grown in the presence of FBS and dashed lines those grown in its absence. IC<sub>50</sub> values were determined from interpolation of the concentration–viability curves and are listed in Table 4.
Supplementary Figure 5: Effects of DIM-Ph-4-Xs and DIM-Ph-4-X+ OM-s on MMTV-Wnt1 murine mammary cancer stem cell viability. Cells were treated for 72 h with each compound at the indicated concentrations in DMSO or with DMSO alone (control, 0.1% final concentration) before the number of viable treated cells relative to vehicle-treated control cells was determined as described in the Methods. Data points represent the means of triplicates ± SD of the ratios of treated to control cell levels × 100. Concentration–viability response curves for DIM-Ph-4-Xs are represented by dashed lines and open symbols and those for DIM-Ph-4-X+ OM-s by solid lines and solid symbols. Individual compounds are designated by their X substituent (X = CF₃, CO₂Me, Cl, OMe and CO₂H). IC₅₀ values for 50% viability reduction listed in Table 1 were determined by interpolation of these curves.
Supplementary Figure 6: The cation (DIM-Ph-4-CF₃⁺) of DIM-Ph-4-CF₃⁺ OMs⁻ docks to the two allosteric binding sites of the NR4A1 LBD protein structure (PDB 3V3Q). (A) Low-energy conformation of DIM-Ph-4-CF₃⁺ docked to allosteric site 1 with adjacent site (LBP) helices H1, H3, H5, H7 and H8 shown in magenta. (B) Low-energy conformation of DIM-Ph-4-CF₃⁺ docked to allosteric site 2 with adjacent site helices H3, H4, H5, H11 and H12 shown in green. The allosteric site surfaces around DIM-Ph-4-CF₃⁺ together with key helices in sites 1 and 2 within 8 Å are shown. The pose for DIM-Ph-4-CF₃⁺ is shown in stick format with C atoms in yellow, N in blue, and F in light-blue. Allosteric site surface N atoms are colored in blue; Os in red. H atoms were omitted for clarity. Docking studies employed GOLD software in the Discovery Studio 3.5 package.

Supplementary Figure 7: LNCaP-SKP2 cells maintain sSupplementary Table KP2 overexpression. (A) Each of the five monoclonal LNCaP cell lines single cell clones from SKP2 overexpressing polyclonal lines were tested for stable overexpression of SKP2 by Western blotting. LNCaP parental cells and LNCaP cells transfected with pcDNA3.1 only were used as controls. p27 expression was found downregulated in clone #1-3. Clone #2 was ultimately used for experiments due to the high level of overexpression of SKP2 and the downregulation of p27. (B) Stable LNCaP-SKP2 cells were treated with increasing doses of SMIP004-7 for 96 hours. Cell viability assay determined that LNCaP-SKP2 cells are highly sensitive to SMIP004-7 (IC₅₀ 0.3 μM) while LNCaP cells stably transfected with empty pcDNA3.1 are less sensitive (IC₅₀ of 3.5 μM).
Supplementary Figure 8: Specificity of NR4A1 antibodies and subcellular localization of NR4A1. (A) 293T cells were transfected with either siRNA targeting Nur77 (NR4A1) or control siRNA as described in the methods. Knockdown efficiency and specificity was analyzed by immunofluorescence staining with NR4A1 XP® monoclonal antibody (Cell Signaling Technologies) as described in the methods. Immunofluorescence staining demonstrated that the NR4A1 XP® monoclonal antibody specifically recognizes NR4A1. Immunofluorescence results were confirmed with another antibody (NR4A1 polyclonal antibody, Sigma). (B) The same tests performed in (A) were done in MCF7 cells. (C) The same tests performed in (A) were done in LNCaP-SKP2 cells. (D) NR4A1 knockdown efficiency in LNCaP-SKP2 cells was determined by immunoblotting with NR4A1 XP® monoclonal antibody. ACTIN is shown for reference. (E) Nuclear and cytoplasmic fractions from LNCaP-SKP2 and MCF7 cells were analyzed by immunoblotting with the NR4A1 XP® monoclonal antibody. Signals obtained with nuclear marker PARP and cytoplasmic tubulin are shown for reference. (F) 293T cells were transfected with either siRNA targeting Nur77 (NR4A1), myc-TR3 plasmid DNA that overexpresses TR3 (NR4A1) or both. NR4A1 XP® monoclonal antibody (Cell Signaling Technologies) specificity was further validated through immunofluorescence staining and analysis.
Monitoring the Oxidation of DIM-Ph-4-Xs (X = CF₃ and CO₂Me) to DIM-Ph-4-X⁺ OMs’s

(X = CF₃ and CO₂Me)

Compound purity analyses

Reversed-phase HPLC analysis of target compounds in Supplementary Table 2 was performed on a Shimadzu 20A HPLC system using elution from a 4.6-mm × 150-mm TITAN C₃₈ column (5-μm particle size), with the UV absorbance detection at 254 nm, and the mixed A/B solvent system described below at a flow rate of 1.0 mL/min:

Solvent A: 0.2% H₃PO₄ in H₂O
Solvent B: 0.2% H₃PO₄ in MeOH

Using the solvent gradient 35 min 70% A/30% B to 5% A/95% B (0–20 min) followed by 95% B (20–33 min) and then 5% A/95% B to 70% A/30% B (2-min recycle).

Reported leukemia cell mutations, gene transpositions and over or under-expressed proteins

DIM-Ph-4-CF₃⁺ docks to NR4A1 LBD structure 3V3Q

Although the pose in Figure S6A shows that the 4-CF₃Ph group and benzo portions of the indole rings of the cation are more exposed on the LBD surface, a partial π–π stacking between its phenyl ring and that of the H5 Tyr122 backbone C = O and the helix H7 His163 backbone C = O and N, respectively. A partial π–π stacking interaction could occur between the phenyl rings of Csn-B and the H5 Tyr122.

Docking of Csn-B to the apo-NR4A1 LBD (PDB 2QW4)

Our docked pose for Csn-B showed that its 2-octanoyl side chain could extend into the interior of the allosteric pocket to form van der Waals contacts with several pocket residues (Figure 3E). Its 3-OH and 5-OH groups could form H-bonds with the loop L7–8 Val167 backbone C = O and the helix H7 His163 backbone C = O and N, respectively. A partial π–π stacking interaction could occur between the phenyl rings of Csn-B and the H5 Tyr122.

Supplemental synthetic methods and target compound characterizations

General methods

Chemicals and solvents were obtained from commercial sources and used without purification. Unless mentioned, anhydrous and/or oxygen-sensitive reactions were carried out under argon gas. Reactions were monitored by thin-layer chromatography on silica gel (mesh size 60, F₂₅₄) with visualization under UV light. Unless otherwise specified, the standard work-up involved washing the organic extract with water and brine and then drying it over anhydrous sodium sulfate followed by filtration and concentration under reduced pressure. Chromatography refers to flash column chromatography on silica gel (Merck 60, 230-400 mesh). Most experimental procedures were not optimized. Melting points of compounds were determined in capillary tubes using a Mel-Temp II apparatus and were not corrected. Infrared spectra of powdered or liquid samples were obtained using an FT-IR Mason satellite spectrophotometer. Unless mentioned, ¹H and ¹³C NMR spectra were obtained on compounds dissolved in CDCl₃ or the specified solvent using a 300-MHz Varian Unity Inova or a 400-MHz ECS Jeol spectrometer. Proton chemical shifts are expressed in ppm (δ) relative to CHCl₃ as the internal standard. High-resolution mass spectra were determined using an Agilent Technologies 6224A accurate mass TOF LC/MS system at Sanford-Burnham Medical Research Institute (Lake Nona, FL). A Shimadzu HPLC system was used to analyze the oxidation rate of DIM-Ph-4-CF₃ to DIM-Ph-4-CF₃⁺ OMs (Supplementary Table 1) and the purity of target molecules (Supplementary Table 2). The purity of compounds used in biological assays was ≥ 95% as determined by HPLC. Compound nomenclature used in these Methods follows that currently employed by Chemical Abstracts.

Condensation of indole with 4-substituted benzaldehydes produces the corresponding 4-substituted di(1H-indol-3-yl)methylbenzenes (DIM-Ph-4-Xs)

A reported procedure was used to generate the CeCl₃•7 H₂O–NaI–SiO₂ catalyst [9]. Briefly, to a solution of CeCl₃•7 H₂O (0.9 mmol) and NaI (0.9 mmol) in CH₃CN (21 mL) was added silica gel (13.9 mmol). This mixture was stirred for approximately 21 h before the solvent was removed at reduced pressure to yield a yellow solid. To this solid was added CH₃CN (7.5 mL), indole (6.0 mmol) and the 4-substituted benzaldehyde (3.0 mmol).
The mixture was stirred for 22–52 h, diluted with Et₂O (100 mL) and filtered through a short pad of Celite® (Et₂O rinse). The filtrate was concentrated at reduced pressure, and the residue was purified by chromatography to give the 4-substituted di(1H-indol-3-yl)methylbenzene (DIM-Ph-4-X).

3.3''-[(4-Trifluoromethyl)phenyl)methylene]bis(1H-indole) (DIM-Ph-4-CF₃) [41]

4- (Trifluoromethyl)benzaldehyde (1.05 g, 6.0 mmol) and indole (1.41 g, 12.0 mmol) after reaction for 48 h, work-up and chromatography (22–25% EtOAc/hexane) produced 2.38 g (92%) of DIM-Ph-4-CF₃ as a white solid, mp 188–190° C. IR 3410, 3054, 1508, 1455, 1243, 1092, 742 cm⁻¹; ¹H NMR δ 5.94 (s, 1H, ArCH), 6.66 (d, J = 1.36 Hz, 2H, 2-IndoleH), 6.99–7.04 (m, 2H, 5-IndoleH), 7.17–7.21 (m, 2H, 6-IndoleH), 7.34–7.38 (m, 4H, 4,7-IndoleH), 7.45 (d, J = 8.7 Hz, 2H, 2,6-ArH), 7.52 (d, J = 8.7 Hz, 2H, 3,5-ArH), 7.95 ppm (bs, 2H, NH). The ¹H NMR data are in agreement with those reported [40]; ¹³C NMR (CDCl₃) δ 130.23, 129.52, 128.98, 130.92, 122.16, 123.62, 123.69, 125.19, 126.80, 128.98, 135.22, 136.66, 148.11 ppm. The Safe group had synthesized DIM-Ph-4-CF₃ using acetic acid [41] rather than CeCl₃·7 H₂O–NaI–SiO₂ as the catalyst.

3.3''-[(4-Methoxyphenyl)methylene]bis(1H-indole) (DIM-Ph-4-OMe) [41]

4-Methoxybenzaldehyde (417 mg, 3.0 mmol) and indole (703 mg, 6.0 mmol) after reaction for 48 h, work-up and chromatography (22–25% EtOAc/hexane) produced 780 mg (74%) of DIM-Ph-4-OMe as a white solid, mp 188–190° C. IR 3410, 3054, 1508, 1455, 1243, 1092, 742 cm⁻¹; ¹H NMR δ 5.81 (s, 1H, ArCH), 6.78 (d, J = 2.3 Hz, 2H, 2-IndoleH), 7.34–7.38 (m, 4H, 4,7-IndoleH), 7.45 (d, J = 8.7 Hz, 2H, 2,6-ArH), 7.52 (d, J = 8.7 Hz, 2H, 3,5-ArH), 7.95 ppm (bs, 2H, NH). The ¹H NMR data are in agreement with those reported [40]; ¹³C NMR (CDCl₃) δ 130.23, 129.52, 128.98, 130.92, 122.16, 123.62, 125.19, 126.80, 128.98, 135.22, 136.66, 148.11 ppm. The Safe group had synthesized DIM-Ph-4-OMe using acetic acid [41] rather than CeCl₃·7 H₂O–NaI–SiO₂ as the catalyst.

Methyl 4-[di(1H-indol-3-yl)methyl]benzoate (DIM-Ph-4-CO₂Me) [42]

Methyl 4-formylbenzoate (493 mg, 3.0 mmol) and indole (703 mg, 6.0 mmol) after reaction for 52 h, work-up and chromatography (25–33% EtOAc/hexane) produced 1.02 g (89%) of DIM-Ph-4-CO₂Me as a white solid, mp 86–89° C. IR 3411, 3055, 1706, 1456, 1283, 1110, 741 cm⁻¹; ¹H NMR δ 3.78 (s, 3H, OCH₃), 5.84 (s, 1H, ArCH), 6.66 (d, J = 2.3 Hz, 2H, 2-IndoleH), 6.82 (t, J = 7.3 Hz, 2H, 5-IndoleH), 7.00 (t, J = 7.3 Hz, 2H, 6-IndoleH), 7.22 (d, J = 7.8 Hz, 2H, 7-IndoleH), 7.45 (d, J = 8.3 Hz, 2H, 2,6-ArH), 7.83 (d, J = 8.3 Hz, 2H, 3,5-ArH), 10.83 ppm (s, 2H, NH).

3.3''-(4-Chlorophenyl)methylene]bis(1H-indole) (DIM-Ph-4-Cl) [42]

4-Chlorobenzaldehyde (435 mg, 3.0 mmol) and indole (703 mg, 6.0 mmol) after reaction for 29 h, work-up and chromatography (20–25% EtOAc/hexane) produced 1.03 g (96%) of DIM-Ph-4-Cl as a white solid, mp 83–86° C. IR 3412, 3054, 1487, 1337, 1089, 742 cm⁻¹; ¹H NMR δ 5.84 (s, 1H, ArCH), 6.78 (d, J = 2.3 Hz, 2H, 2-IndoleH), 6.83 (t, J = 7.3 Hz, 2H, 5-IndoleH), 7.00 (t, J = 7.3 Hz, 2H, 6-IndoleH), 7.22 (d, J = 7.8 Hz, 2H, 4-IndoleH), 7.26–7.33 (m, 6H, 7-IndoleH, 2,6-ArH, 3,5-ArH), 10.80 ppm (s, 2H, NH).

4-[Di(1H-indol-3-yl)methyl]benzoic acid (DIM-Ph-4-CO₂H) [42]

To a stirred solution of DIM-Ph-4-CO₂Me (700 mg, 1.84 mmol) in MeOH (20 mL) was added 5 M aq NaOH (1.84 mL, 9.20 mmol). This mixture was heated at reflux under argon for 50 min, cooled to room temperature and acidified (1 N HCl, 40 mL) and extracted (EtOAc, 130 mL). The organic extract was washed (brine) and dried. The residue obtained on concentration was washed (pentane and CH₂Cl₂) to afford 590 mg (88%) of DIM-Ph-4-CO₂H as a white solid, mp 249–250° C. IR 3410, 3051, 1697, 1430, 1382, 742 cm⁻¹; ¹H NMR (CDCl₃) δ 5.92 (s, 1H, ArCH), 6.65 (d, J = 2.3 Hz, 2H, 2-IndoleH), 6.87 (t, J = 7.5 Hz, 2H, 5-IndoleH), 7.04 (t, J = 7.5 Hz, 2H, 6-IndoleH), 7.25 (d, J = 8.2 Hz, 2H, 4-IndoleH), 7.32 (d, J = 8.2 Hz, 2H, 7-IndoleH), 7.43 (d, J = 8.3 Hz, 2H, 2,6-ArH), 7.92 ppm (d, J = 8.3 Hz, 2H, 3,5-ArH).

Oxidation of di(1H-indol-3-yl)methyl(4-X-benzene) to their di(1H-indol-3-yl)(4-X-phenyl) methylum mesylates

A reported procedure for the synthesis of tris(1-alkylindol-3-yl)methylum salts [4] was adapted. To a solution of the di(1H-indol-3-yl)(4-X-phenyl)methane (0.5 mmol) in 1-butanol (5 mL) was added activated carbon powder (Norit®, 10–25 mg) and MeSOH (1.5 mmol). This mixture was stirred for more than 20 h under air and then filtered through a short pad of Celite®. The filtrate was washed (water) and concentrated under reduced pressure. The brown residue was triturated (Et₂O), collected by filtration using a sintered glass filter (Et₂O wash) and dried at reduced pressure. The resultant solid was washed (Et₂O, 4f) and dried to give the product as a reddish-brown powder. Replacing 1-butanol by 2-butanol gave a similar yield.
Di(1H-indol-3-yl)(4-trifluoromethylphenyl) methylimy mesylate

(DIM-Ph-4-CF3OMs)

Procedure A. DIM-Ph-4-CF3 (195 mg, 0.5 mmol), MsOH (97 µL, 1.5 mmol) and activated carbon powder (10 mg, 0.8 mmol) in 1-butanol after reaction for 21 h, work-up and purification produced 38 mg (16%) of DIM-Ph-4-CF3OMs as a reddish-brown powder, mp 245–248°C (dec). IR 3115, 2943, 1488, 1413, 1178, 1120, 757 cm−1; 1H NMR (DMSO-d6) δ 2.30 (s, 3H, CH3SO2); 3.96 (s, 3H, OCH3); 7.18 (t, J = 6.8 Hz, 2H, 5-IndoleH), 7.40 (t, J = 6.8 Hz, 2H, 6-IndoleH), 7.70 (d, J = 7.4 Hz, 2H, 4-IndoleH), 7.89 (d, J = 6.8 Hz, 2H, 2-IndoleH); 8.05 (d, J = 6.9 Hz, 2H, 2,6-ArH); 8.15 ppm (bs, 2H, 2,6-ArH); 8.28 (d, J = 8.2 Hz, 2H, 2,6-ArH); 8.31 ppm (bs, 2H, 2-IndoleH). HRMS calcd C23H16F3N2O [M + H]+ 389.1259, found 389.1260. Procedure B. Pure O2 was bubbled for 46 h into a stirred mixture of DIM-Ph-4-CF3 (49 mg, 0.125 mmol), MsOH (24 µL, 0.375 mmol) and activated carbon powder (8 mg, 0.6 mmol) in 1-butanol. The same work-up and purification as in Procedure A produced 22 mg (6%) of DIM-Ph-4-CF3OMs as a reddish-brown powder.

Di(1H-indol-3-yl)(4-methoxycarbonylphenyl) methylimy mesylate

(DIM-Ph-4-CO2MeOMs)

Procedure A. DIM-Ph-4-CO2Me (190 mg, 0.5 mmol) and activated carbon powder (25 mg, 2.1 mmol) in 1-butanol after reaction for 43 h under air, work-up and purification produced 31 mg (13%) of DIM-Ph-4-CO2MeOMs as a brown powder, mp 247–250°C (dec). IR 3118, 2989, 1732, 1488, 1416, 1197, 1122, 752 cm−1; 1H NMR (CD3OD) δ 2.68 (s, 3H, CH3SO2), 4.01 (s, 3H, OCH3), 6.83 (d, J = 6.9 Hz, 2H, 7-IndoleH), 7.16 (t, J = 7.3 Hz, 2H, 5-IndoleH), 7.42 (t, J = 7.3 Hz, 2H, 6-IndoleH), 6.88 (d, J = 8.2 Hz, 2H, 4-IndoleH), 7.79 (d, J = 8.2 Hz, 2H, 2,6-ArH), 8.29 (d, J = 8.2 Hz, 2H, 3,5-ArH), 8.38 ppm (bs, 2H, 2-IndoleH). HRMS calcd C23H18NO2 [M + H]+ 379.1441, found 379.1441. Procedure B. Pure O2 was bubbled for 46 h into a stirred mixture of DIM-Ph-4-CO2Me (48 mg, 0.125 mmol), MsOH (24 µL, 0.375 mmol) and activated carbon powder (8 mg, 0.6 mmol) in 1-butanol. The same work-up and purification as in Procedure A produced 19 mg (32%) of DIM-Ph-4-CO2MeOMs as a reddish-brown powder.

Di(1H-indol-3-yl)(4-chlorophenyl)methylimy mesylate (DIM-Ph-4-ClOMs)

DIM-Ph-4-Cl (357 mg, 1.0 mmol) and activated carbon powder (50 mg, 0.42 mmol) in 1-butanol after reaction for 45 h under air, work-up and purification produced 123 mg (27%) of DIM-Ph-4-ClOMs as a brown powder, mp 193–196°C (dec). IR 3108, 2883, 1484, 1412, 1175, 1117, 751 cm−1; 1H NMR (CD3OD) δ 2.69 (s, 3H, CH3SO2), 6.90 (d, J = 8.2 Hz, 2H, 7-IndoleH), 7.16 (t, J = 7.3 Hz, 2H, 5-IndoleH), 7.42 (t, J = 7.3 Hz, 2H, 6-IndoleH), 7.65–7.72 (m, 6H, 4-IndoleH, 2,6-ArH, 3,5-ArH), 8.31 ppm (bs, 2H, 2-IndoleH). HRMS calcd C23H16ClN2O [M + H]+ 355.0997, found 355.0969.

Di(1H-indol-3-yl)(4-methoxyphenyl)methylimy mesylate (DIM-Ph-4-OMeOMs)

DIM-Ph-4-OMe (264 mg, 0.75 mmol) and activated carbon powder (40 mg, 3.3 mmol) in 1-butanol after reaction for 30 h under air, work-up and purification produced 97 mg (27%) of DIM-Ph-4-OMeOMs as a brown powder, mp 247–250°C (dec). IR 3109, 2933, 1483, 1412, 1171, 1123, 746 cm−1; 1H NMR (CD3OD) δ 2.63 (s, 3H, CH3SO2), 3.96 (s, 3H, OCH3), 6.90 (d, J = 7.8 Hz, 2H, 7-IndoleH), 7.01 (t, J = 7.3 Hz, 2H, 5-IndoleH), 7.16 (d, J = 7.8 Hz, 2H, 3,5-ArH), 7.26 (t, J = 7.3 Hz, 2H, 6-IndoleH), 7.49 (d, J = 7.8 Hz, 2H, 4-IndoleH), 7.59 (d, J = 7.8 Hz, 2H, 2,6-ArH), 7.95 ppm (bs, 2H, 2-IndoleH). HRMS calcd C23H16NO2 [M + H]+ 351.1492, found 351.1467.

Di(1H-indol-3-yl)(4-carboxyphenyl)methylimy mesylate (DIM-Ph-4-CO2HOMs)

DIM-Ph-4-CO2H (275 mg, 0.75 mmol) and activated charcoal (40 mg, 3.3 mmol) in 1-butanol after reaction for 120 h under air, work-up and purification produced 22 mg (6%) of DIM-Ph-4-CO2HOMs as a brown powder mp >260°C (dec). IR 2960, 2866, 1484, 1413, 1174, 1124, 1054, 668 cm−1; 1H NMR (CD3OD) δ 2.69 (s, 3H, CH3SO2), 6.84 (bs, 2H, 7-IndoleH), 7.18 (t, J = 7.8 Hz, 2H, 5-IndoleH), 7.42 (t, J = 7.8 Hz, 2H, 6-IndoleH), 7.68 (d, J = 8.2 Hz, 2H, 4-IndoleH), 7.78 (d, J = 8.2 Hz, 2H, 2,6-ArH), 8.28 (d, J = 8.2 Hz, 2H, 3,5-ArH), 8.39 ppm (bs, 2H, 2-IndoleH). HRMS calcd C23H17N2O2 [M + H]+ 365.1258, found 365.1259.

Oxidation of 4-Substituted Di(1H-indol-3-yl) methylbenzenes to (E)-3-[(1H-indol-3-yl)(4-X-phenyl)methylene]-3H-indoles (X = CF3 and CO2Me)

A reported method [6] was used. To a stirred solution of the DIM-Ph-4-X in MeCN was added 2,3-dichloro-
5,6-dicyano-p-benzoquinone (DDQ) and stirring was continued at room temperature for 2–3 h. Chromatography produced a dark-red solid, which was washed with EtOAc (X = CF₃) or MeCN (X = CO₂Me) and then with ether. The crude product was purified by chromatography to give the I(Ph-4-X)MI.

(E)-3-((1H-indol-3-yl)(4-trifluoromethylphenyl)methylene)-3H-indole (I(Ph-4-CF₃)MI)

DIM-Ph-4-CF₃ (195 mg, 0.5 mmol) and DDQ (136 mg, 0.6 mmol) after reaction for 2 h 10 min, work-up and chromatography (5–10% MeOH/CH₂Cl₂) produced 147 mg (76%) of I(Ph-4-CF₃)MI as an orange powder, mp 241–244°C (dec). IR (CHCl₃) 3084, 2914, 2808, 1548, 1475, 1415, 1322, 1133, 1103, 1064, 739 cm⁻¹; ¹H NMR (DMSO-d₆) δ 6.58 (bs, 2H, 7-IndoleH), 6.94 (t, J = 7.3 Hz, 2H, 5-IndoleH), 7.20 (t, J = 7.3 Hz, 2H, 6-IndoleH), 7.54 (d, J = 7.8 Hz, 2H, 4-IndoleH), 7.69 (bs, 2H, 2,6-ArH), 7.91 (d, J = 7.8 Hz, 2H, 3,5-ArH), 8.02 ppm (bs, 2H, 3,5-ArH).

Di(1H-indol-3-yl)(4-methoxycarbonylphenyl)methyl mesylate

(DIM-Ph-4-CO₂MeOMs⁻)

I(Ph-4-CO₂Me)MI (23 mg, 0.06 mmol) and MsOH (32 µL, 0.24 mmol) after reaction for 3 h 10 min and work-up produced 19 mg (65%) of DIM-Ph-4-CO₂MeOMs⁻ as a reddish-brown powder. IR (CHCl₃) 1171 cm⁻¹ (broad, S = O stretch). The ¹H NMR spectrum of this product (DMSO-d₆) was identical to that obtained by the oxidation of Ph-4-CF₃ using O₂/activated C/MeSO₃H method.

Di(1H-indol-3-yl)(4-methoxycarbonylphenyl)methyl mesylate

(DIM-Ph-4-CO₂MeOMs⁻)

Experiments were performed at a scanning rate of 1 K/min under 3.0 atm of pressure using an N-DSC II differential scanning calorimeter (Calorimetry Sciences Corp.). Samples contained N4R4A1 LBD (20 µM, 0.51 mg/ml) alone or with compound (30 or 60 µM), and 5% DMSO in PBS, pH 7.4. The reference contained 5% DMSO in PBS. Data were analyzed using the NanoAnalyse software package (TA Instruments). Melting temperature (Tₘ) corresponds to the maximum thermal transition temperature. Calorimetric enthalpy (ΔH) was calculated as the area under the excess heat capacity function (Cₚ).

Computational methods

Volume calculation

Chemical group volumes (CO₂Me, 53.6 Å³; CF₃, 40.3 Å³; CO₂H, 36.0 Å³; OMe, 34.6 Å³; and Cl, 22.6 Å³) were calculated using the free online service for calculating molecular properties provided by Molinspiration Cheminformatics (Nova ulica, Slovensky Grob, Slovak Republic) [7].

Molecular modeling

Docking of the cation (DIM-Ph-4-CF₃⁺) of DIM-Ph-4-CF₃OMs⁻ into the pocket corresponding to allosteric
site 1 in the crystal structure of the human NR4A1 (TR3) LBD protein (PDB 2QW4) [43] employed BioMed Cache vs. 6.2 software, which is no longer supported by Fujitsu Limited, and our previously described methods [44]. Briefly, the LBP was derived by selecting all neighboring residues within a 10-Å radius to ensure that all residues were encompassed. In the docking process, the energy-minimized pose of Csn-B was kept rigid to maintain the geometry used for docking by Wu and colleagues [45], whereas the carbocation was allowed to be flexible. In both cases, the side chains of pocket residues were allowed to be flexible. The docked poses for Csn-B and DIM-Ph-4-CF$_3^+$ (Figure 3E) were analyzed by measuring inter-atom distances after superposing the helical backbones of the docked NR4A1 LBD poses.

Docking of DIM-Ph-4-CF$_3^+$ into allosteric sites 1 and 2 on the structure (PDB 3V3Q) [11] of the complex of the NR4A1 LBD with two molecules of the Csn-B analogue (structure in Figure S1) used the GOLD docking engine in the Discovery Studio 3.5 package (Accelrys Inc.). The LBP for each site was derived by selecting all neighboring residues within an 8-Å radius of the analogue [11]. The geometry of DIM-Ph-4-CF$_3^+$ was optimized before docking and during docking was allowed to be flexible, whereas the side chains of pocket residues were kept rigid. Possible interactions between DIM-Ph-4-CF$_3^+$ and the residues lining both sites were analyzed and are shown in Figure S6.

Cancer cell line characteristics

HCT-116 colorectal carcinoma cells are poorly differentiated, express a constitutively active mutant p21ras (Ki-ras) proto-oncogene [13] and a mutant β-catenin, which is not inhibited by wild-type APC [14], and over-express c-myc [13]. This cell line also expresses wild-type p53, PPARγ and NR4A1 [12]. Of the breast cancer lines, MCF-7 expresses estrogen receptors and its growth is estrogen-dependent [15, 18], whereas MDA-MB-231 does not express estrogen receptors and its growth is estrogen-independent [18]. Wild-type tumor suppressor p53 and mutant nonfunctional p53 are expressed in MCF-7 and MDA-MB-231 cells, respectively [26]. NR4A1 is expressed in MCF-7 cells [1], but not in MDA-MB-231 cells [1], and PPARγ is expressed in both lines [17, 18]. Of the prostate cancer cell lines, LNCaP cells are androgen-dependent and express the mutant androgen receptor (AR) (Thr877Ala), which is activated by both the androgen dihydrotestosterone (DHT) and the anti-androgen hydroxyflutamide [25]. They also express low levels of PPARγ1 [24] and after treatment with the PPARγ ligand 15d-Pg J$_2$ express modest levels of PPARγ2 [24]. The calcium ionophore A23187, anti-cancer drug etoposide and androgen induce the expression of NR4A1 [27]. LAPC-4 cells are androgen-dependent and express wild-type AR [23, 28]. 22Rv1 cells are reported to grow independently of androgen in vivo and to express both a DHT and hydroxyflutamide-activated full-length AR(His874Tyr) mutant [25] and a truncated 80-KDa AR mutant [29]. They also express NR4A1 [24], but not PPARγ [24]. The expression of NR4A1 and PPARγ in LAPC-4 cells [23] has not been reported. The growth of PC-3 cells, which are AR null, is androgen independent [25]. They weakly express PPARγ1 [25] and after treatment with 15d-Pg J$_2$ robustly express PPARγ2 [25]. A23187 or etoposide treatment induces their expression of NR4A1 [27]. PC-3 cells do not express p53 [26], whereas the other three lines do [26]. The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) enzyme is reported to antagonize AR transactivation and Akt–PI3K signaling, and its loss is associated with advanced prostate cancer [28]. PTEN is expressed and functional in LACP-4 [28] and 22Rv1 [28, 29] cells, although in the former its expression is reported to be low [29], PTEN is absent in LNCaP and PC-3 cells [28].

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