Supporting Information

Synthesis of novel 3,5-disubstituted-2-oxindole derivatives as antitumor agents against human Non-Small Cell Lung Cancer

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Experimental section

1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references ^{1}H NMR and ^{13}C NMR spectra of all compounds were obtained with a Gemini 200 spectrometer operating at 200 MHz, in a ~2% solution of $CDCl_3$, CD_3OD-d_4 and DMSO-d_6. The elemental compositions of the compounds agreed to within ± 0.4% of the calculated value.

Mass spectra were obtained on a Hewlett-Packard 5988 A spectrometer using a direct injection probe and an electron beam energy of 70 eV. Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F_{254}) sheets that were visualised under a UV lamp. Evaporation was performed *in vacuo* (rotating evaporator). Sodium sulfate was always used as the drying agent. Commercially available chemicals were purchased from Sigma-Aldrich.

(3Z)-2-(6,7-dimethoxy-3,4-dihydroisoquinolin-2-(1H)-yl)-N-(2-oxo-3-(thiophen-2-ylmethylene) indolin-5-yl)acetamide **(1a).**

To a solution of the compound **2** (150 mg, 0.39 mmol) in ethanol (7 mL) was added 2-thiophencarboxaldehyde (48 mg, 0.43 mmol) and a catalytic amount of piperidine. The resulting solution was stirred and refluxed for 12 h, then the solution was evaporated to dryness. The residual material was purified by crystallization from AcOEt/ hexane, affording **1a** as the *Z*-isomer (62 mg, 0.13 mmol, 30% yield): mp 158-160°C. ^{1}H NMR ($CDCl_3$): δ 2.90-2.99 (m, 4H, CH_2); 3.34 (s, 2H, CH_2); 3.76 (s, 2H, CH_2); 3.84 (s, 3H, OMe); 3.88 (s, 3H, OMe); 6.54 (s, 1H); 6.66 (s, 1H); 6.77-6.85 (m, 1H, H-C7 indole); 7.15-7.21 (m, 2H, Ar); 7.65 (s, 1H, H-vinyl); 7.76-7.98 (m, 2H, H-C6 indole, Ar); 8.09 (s, 1H, H-C4 indole); 8.82 (br s, 1H, NH); 9.20 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$): δ 170.60, 168.47, 148.03, 147.84, 139.07, 138.05, 137.45, 135.70, 134.02, 132.48, 129.33, 127.60, 125.94, 125.59, 120.06, 116.22, 115.88, 109.77, 62.01, 56.24, 55.97, 51.79, 29.09 ppm. Anal. ($C_{26}H_{25}N_3O_4S$) Calc.% C,65.67; H,5.30; N,8.84. Found%C,65.50; H,4.98; N,8.89.

(3E)-2-(6,7-dimethoxy-3,4-dihydroisoquinolin-2-(1H)-yl)-N-(3-((1-methyl-1H-imidazol-2yl) methylene)-2-oxoindolin-5-yl)acetamide **(1b).**

Compound **1b** was synthesized from **2** (150 mg, 0.39 mmol) and 1-methyl-5-imidazolecarboxaldehyde (47 mg, 0.43 mmol) following the same procedure described above for

the preparation of **1a**. **1b** (*E*-isomer) (71 mg, 0.15 mmol, 38% yield): mp 147-149°C. ^{1}H NMR ($CDCl_3$): δ 2.90-3.00 (m, 4H, CH_2); 3.35 (s, 2H, CH_2); 3.78 (s, 2H, CH_2); 3.84 (s, 3H, NMe); 3.87 (s, 3H, OMe); 3.88 (s, 3H, OMe); 6.55 (s, 1H); 6.67 (s, 1H); 6.82 (d, 1H, *J* = 8.2 Hz, H-C7 indole); 7.05 (s, 1H, Ar); 7.25 (s, 1H, Ar); 7.47 (s, 1H, H-vinyl); 7.73-7.81 (m, 2H, H-C6 indole, NH); 9.27 (br s, 1H, H-C4 indole); 9.38 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$): δ 170.79, 168.53, 148.43, 148.10, 138.49, 132.84, 131.24, 127.00, 126.47, 126.07, 124.34, 122.83, 120.61, 118.71, 112.34, 110.24, 109.66, 62.12, 56.49, 56.09, 51.91, 34.01, 29.35 ppm. Anal. ($C_{26}H_{27}N_5O_4$) Calc.% C, 65.95; H,5.75; N,14.79; Found% C,66.11; H,5.50; N,14.59.

(3Z)-N-(3-((1H-imidazol-5yl)-(2-oxoindolin-5-yl)-2-(6,7-dimethoxy-3,4-dihydroisoquinolin -2-(1H)-yl)acetamide **(1c).**

Compound **1c** was synthesized from **2** (150 mg, 0.39 mmol) and 1H-imidazole-5-carbaldehyde (41 mg, 0.43 mmol) following the same procedure as described above for the preparation of **1a**. **1c** (*Z*-isomer) (73 mg, 0.16 mmol, 42% yield): mp 169-171°C. ^{1}H NMR ($CDCl_3$): δ 2.85-2.95 (m, 4H, CH_2); 3.34 (s, 2H, CH_2); 3.76 (s, 2H, CH_2); 3.85 (s, 3H, OMe); 3.88 (s, 3H, OMe); 6.54 (s, 1H); 6.66 (s, 1H); 6.83 (d, 1H, *J* = 8.2 Hz, H-C7 indole); 7.21-7.28 (m, 1H, H-C6 indole); 7.49 (s, 1H, Ar); 7.60 (s, 1H, H-vinyl); 7.84 (s, 1H, Ar); 7.93 (br s, 1H, H-C4 indole); 8.08 (br s, 1H, NH); 9.23 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$): δ 170.80, 169.58, 148.00, 147.93, 138.99, 135.30, 133.10, 128.67, 125.70, 125.61, 124.90, 123.73, 119.91, 112.01, 111.60, 110.01, 109.92, 62.03, 56.27, 55.90, 51.83, 29.09 ppm. Anal. ($C_{25}H_{25}N_5O_4$) Calc.% C, 65.35; H, 5.48; N, 15.24; Found% C, 65.00; H, 5.39; N, 14.99.

(3E)-2-(6,7-dimethoxy-3,4-dihydroisoquinolin-2-(1H)-yl)-N-(2-oxo-3-(pyridin-2-ylmethylene)indolin-5-yl)acetamide **(1d).**

Compound **1d** was synthesized from **2** (150 mg, 0.39 mmol) and 2-pyridinecarboxaldehyde (46 mg, 0.43 mmol) following the same procedure as described above for the preparation of **1a**. **1d** (*E*-isomer) (84.69 mg, 0.18 mmol, 45% yield): mp 160-172°C. ^{1}H NMR ($CDCl_3$): δ 2.82-2.95 (m, 4H, CH_2); 3.33 (s, 2H, CH_2); 3.70 (s, 2H, CH_2); 3.83 (s, 3H, OMe); 3.87 (s, 3H, OMe); 6.51 (s, 1H); 6.64 (s, 1H); 6.82 (d, 1H, *J* = 8.2 Hz, H-C7 indole); 7.20-7.28 (m, 1H, Py); 7.38-7.52 (m, 2H, Py, H-vinyl); 7.74-7.76 (m, 1H, Py); 7.96-8.10 (m, 2H, Py, H-C6 indole); 8.63 (br s, 1H, NH); 8.88 (br s, 1H, H-C4 indole); 9.08 (br s, 1H) ppm.^{13}C NMR (CD_3OD-d_4): δ 179.08, 176.09, 164.60, 158.52, 151.02, 150.15, 149.10, 148.84, 144.83, 138.33, 127.97, 122.45, 120.34, 118.02, 116.70, 115.07, 113.15, 111.42, 57.52, 56.87, 54.79, 52.33, 25.79 ppm. Anal. ($C_{27}H_{26}N_4O_4$) Calc.% C, 68.92; H, 5.57; N, 11.91; Found% C, 68.99; H, 5.73; N, 11.90.

2-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-N-(2-oxo-2,3-dihydro-1H-indol-5-yl) acetamide **(2).**

A solution of K_2CO_3 (1.19 g, 8.61 mmol) in acetonitrile (8 mL), heated at 80°C, was treated with a solution of compound **4** (879 mg, 3.91 mmol) in DMF (1.3 mL). The suspension was stirred at 80°C for 12 h, then the potassium carbonate was filtered off and the solvent removed. The residue was diluted with CH_2Cl_2 and washed with water and brine. The organic layer was dried and concentrated. The crude product was purified by crystallization from AcOEt/ hexane, affording compound **5** (538 mg, 1.41 mmol, 36 % yield) as a yellow solid. ^{1}H NMR ($CDCl_3$): δ 2.87-2.96 (m, 4H, CH_2); 3.32 (s, 2H, CH_2); 3.52 (s, 2H, CH_2); 3.75 (s, 2H, CH_2); 3.84 (s, 3H, OMe); 3.87 (s, 3H, OMe); 6.53 (s, 1H); 6.64 (s, 1H); 6.79 (d, 1H, *J* = 8.2 Hz, H-C7 indole); 7.27 (dd, 1H, *J* = 2.2, 8.2 Hz, H-C6 indole); 7.62 (d, 1H, *J* = 2.2 Hz, H-C4 indole); 8.10 (br s, 1H, NH); 9.19 (br s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$): δ 177.05, 168.44, 148.23, 147.86, 139.07, 132.82, 126.16, 125.92, 125.59, 119.55, 117.45, 111.96, 109.83, 109.66, 61.94, 56.24, 55.93, 51.76, 36.56, 29.05 ppm. Anal. ($C_{21}H_{23}N_3O_4$) Calc.% C, 66.13; H, 6.08; N, 11.02; Found% C, 66.30; H, 5.89; N, 10.95.

5-amino-1,3-dihydro-2H-indol-2-one **(3).**

The commercial 5-nitro-1,3-dihydro-2*H*-indol-2-one (1.00 g, 5.61 mmol) was hydrogenated in EtOH (70 mL) in the presence of 10% Pd-C (315 mg, 2.97 mmol) for 4 h. Then the catalyst was filtered off, and the solution was evaporated, to give **3** (698 mg, 4.71 mmol, 84% yield). ^{1}H NMR (DMSO-d_6): δ 3.53 (s, 2H, CH_2); 6.82 (d, 1H, *J* = 8.1 Hz, H-C7 indole); 7.05-7.11 (m, 2H, H-C4, H-C6 indole); 10.47 (br s, 1H, NH) ppm.

2-chloro-N-(2-oxo-2,3-dihydro-1H-indol-5-yl)acetamide **(4).**

To a stirred solution of 5-amino-2-oxindole **3** (122 mg, 0.82 mmol) in acetone (5 mL) and DMF (1.2 mL), cooled to 0°C, was added dropwise 2-chloroacetyl chloride (93 mg, 0.82 mmol). The reaction mixture was stirred at room temperature for 3 h, then the solvent was evaporated affording to compound **4** (180 mg, 0.80 mmol, 98% yield). ^{1}H NMR (DMSO-d_6): δ 3.47 (s, 2H, CH_2); 4.21 (s, 2H, CH_2Cl); 6.73 (d, 1H, *J* = 8.4 Hz, H-C7 indole); 7.33 (d, 1H, *J* = 8.4 Hz, H-C6 indole); 7.49 (s, 1H, H-C4 indole); 10.15 (br s, 1H, NH); 10.33 (br s, 1H, NH) ppm.^{13}C NMR (DMSO-d_6): δ 175.92, 163.90, 139.70, 132.22, 125.95, 118.82, 116.61, 108.73, 43.36, 35.90 ppm. Anal. ($C_{10}H_9ClN_2O_2$) Calc% C, 53.47; H, 4.04; N, 12.47; Found% C, 53.51; H, 4.32; N, 12.66.

2. Biology

2.1 Drugs and chemicals

Perifosine (Keryx Biopharmaceuticals, Inc.), an Akt inhibitor currently evaluated as an anti-cancer agent in phase 1 and 2 clinical trials[1], was used as reference drug. Perifosine and test compounds were dissolved in PBS and DMSO, respectively, and then stored at -20 °C until use. All compounds were diluted in culture medium immediately before use. RPMI and DMEM media, foetal bovine serum, horse serum, l-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin (50 microg/ml) were from Life Technologies, Inc. (Gaithersburg, Md., USA). All other chemicals were from Sigma (St. Louis, Mo., USA).

Anti-phospho-EGFR (Tyr992) antibody, anti-phospho-Her2 (Tyr1221/1222), anti-phospho-FoxO1 (Thr24)/FoxO3a (Thr32), anti-phospho-Akt (Thr308), anti-Akt and anti FoxO1 were purchased from Cell Signaling Technology (Beverly, MA). Anti-Actin was purchased from Santa Cruz Biotechnology, USA

2.2 Cell lines

The human cancer cell lines A549, HCC827 (lung) and MDA-MB-231 (breast) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI (A549 and HCC827) or L-15 (MDA-MB-231) with 10% fetal bovine serum, 1% glutamine and 1% penicillin–streptomycin. Cells were cultured in 75-cm^2 flasks (Costar, Cambridge, MA, USA), at 37 °C in 5% CO_2 and 95% air, and were harvested with EDTA when they were in logarithmic growth. The MDA-MB-231 cells were grown in L-15 with 10% fetal bovine serum.

2.3 Cell proliferation assay

A549 or HCC827 cells were plated in 24-well sterile plastic plates (Sarstedt) at 2×10^4 cells/well, allowed to attach for 24 h, and then treated with compounds at 0.01–100 μM for 24, 48 and 72 hours. At the end of drug exposure, cells were harvested and counted by haemocytometer and changes in cell growth were expressed as a percentage relative to untreated cells. The half maximal inhibitory concentration (IC_{50}) was calculated by non-linear least squares curve fitting.

MDA-MB-231 cells were plated in 12-well sterile plastic plates, allowed to attach for 24 h, and then treated in duplicate with indicated compounds at 1 and 10 μM. After 72 hours, the number of cells was assessed by manual cell counting. Changes in cell growth were expressed as a percentage relative to cells treated with DMSO.

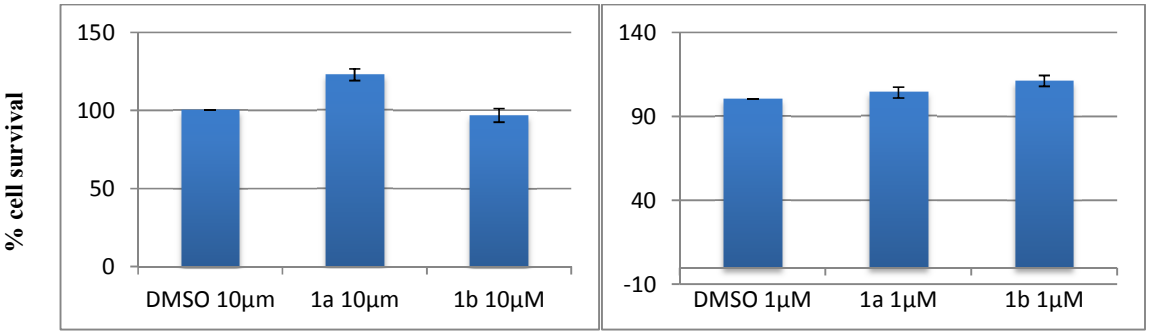

Figure S1. Effect of compounds **1a** and **1b** on MDA-MB-231 cell proliferation. Data were expressed as mean values ± SEM (n=3).

2.4 Cell-cycle analysis

After treatment with compounds at their IC50s followed by a 24 h washout, cells were harvested with tripsin/EDTA and washed with PBS. Cells were then fixed in cold 70% ethanol added dropwise to the pellet while vortexing, to ensure fixation of all cells and minimize clumping. Cells were allowed to fix for 30 min at 4°C and then washed twice in PBS and spun at 850g in a centrifuge to remove ethanol by discarding the supernatant. Thereafter, cells were treated with ribonuclease by adding 50 µl of a 100 µg/ml stock solution of RNase to ensure that only DNA is stained. Finally, 10 µl of 1 mg/ml propidium iodide solution (final concentration being 10 µg/ml) were added and cells stored in the dark and at 4°C until analysis onto FACS by reading on cytometer at 488 nm. Cytofluorimetry was performed using a FACScan (BD Biosciences, San Jose, CA) and data analysis was carried out with CELLQuest software, whereas the cell-cycle distribution was determined using ModFit software (Verity Software House, Topsham, ME).

2.5 Analysis of apoptosis

Cells were treated with compounds at their IC50s and, at the end of incubation, washed twice with PBS and fixed in 4% buffered p-formaldehyde for 15 min. Cells were then spotted on glass slides and examined by fluorescence microscopy (Leica, Wetzlar, Germany, DE). A total of 200 cells from randomly selected microscopic fields were counted and the percentage of cells displaying chromatin condensation and nuclear fragmentation relative to the total number of counted cells (apoptotic index) were calculated. Apoptosis induced by compounds was also studied by flow cytometry.

2.6 Akt activation assay

A specific ELISA method was used to analyze Akt phosphorylation on the threonine and serine residues at position 308 (pThr308) and 473 (pSer473), respectively, following the manufacturer's

protocol (BioSource International, Camarillo, CA). Quantitation of pThr308 Akt and pSer473 Akt was obtained by standard curve and values normalized for total Akt and protein content (Lowry reagent, Sigma Chemical Co. St Louis, USA).

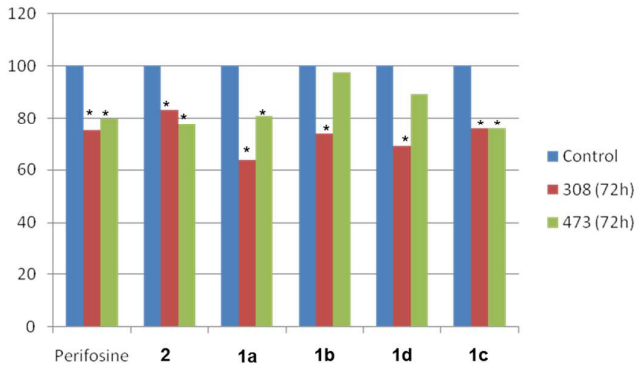

Figure S2. Effect of test compounds and the Akt inhibitor perifosine on the phosphorylation of pThr308 and pSer473 Akt in A549 cells after 72 h. Data were expressed as mean values ± S.D (n=3). *P < 0.05, as compared with control

2.7. *Statistical analysis*

All experiments were performed in triplicate and repeated three times. Data were expressed as mean values ± S.D. and analyzed by Student's t test or ANOVA as appropriate; the level of significance was set at $P < 0.05$.

2.8. *Western Blot Analysis*

To examine the basal condition phosphorylation of proteins, confluent cells in 6-well dishes were cultured overnight in serum-free medium. The A549 cells were pre-treated with OXIDs at 10 μM concentration in serum-free medium for 3 h before treating with the same compounds dissolved in medium supplemented with 10% FBS for 10 min at 37°C.

The cells were then washed with ice-cold PBS and lysed in lysis buffer containing 2%SDS in PBS and the protein concentration was determined using a Pierce BCA protein assay kit (Thermo Scientific). 10-25 μg of proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, USA). Membranes were probed with the indicated primary antibodies overnight at 4°C and then visualized with HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL, Amersham).

Phosphorylation levels of EGFR, Akt threonine 308, and FoxO 1-3a were analysed. Equal loading was determined using an anti-actin antibody. Phosphorylation of Her2 in the same lysates was analysed in parallel gels. Anti-actin was used as equal loading control. Akt 1/2/3 and FoxO1 levels were determined after stripping of membranes previously incubated with the anti-phosphoThr308 Akt and anti-phospho FoxO 1-3a.

Band densitometry was performed using ImageJ software (NIH, Bethesda, USA). Phospho Akt308 and Phospho FoxO 1-3a, phospho EGFR and phospho Her2 were normalized to actin as a loading control.

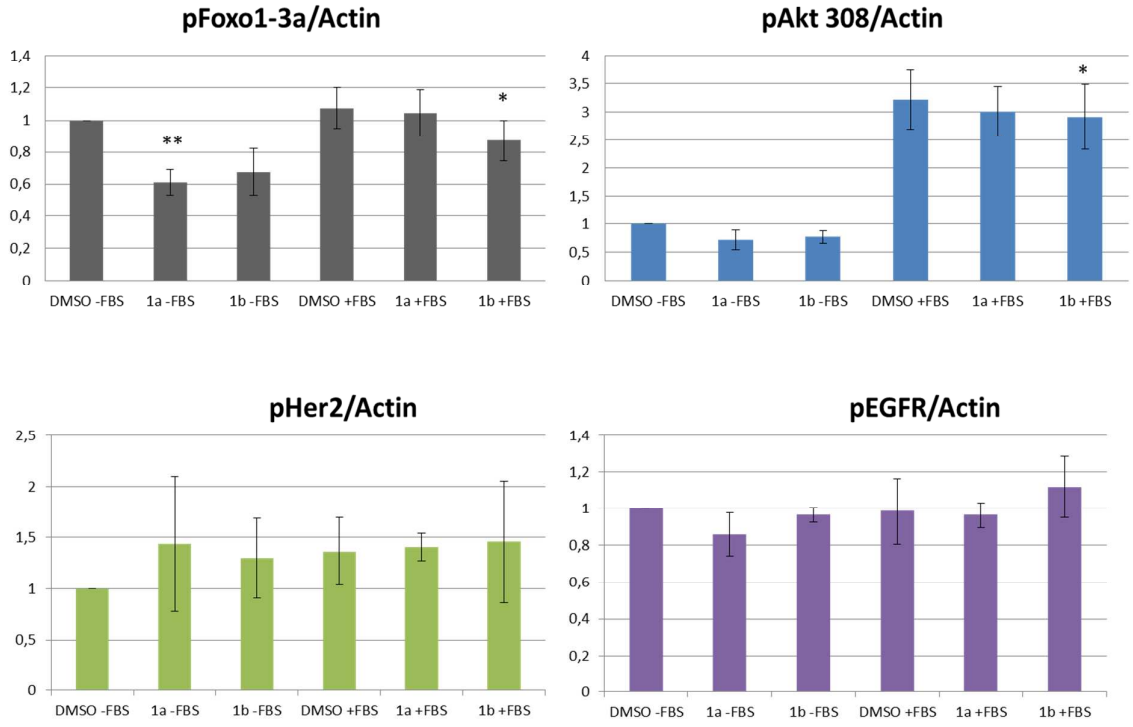

Figure S3. Band densitometry. Data were expressed as mean values ± SEM (n=3). Significance was calculated with Student's t tests (paired samples, one tailed, $p < 0.059$).

3. *References*

1. Pinton G, Manente AG, Angeli G, Mutti L, Moro L. Perifosine as a potential novel anti-cancer agent inhibits EGFR/MET-AKT axis in malignant pleural mesothelioma. PLoS One. 2012;7(5):e36856.