Supporting Information

Discovery of *O*-Alkylamino Tethered Niclosamide Derivatives as Potent and Orally Bioavailable Anticancer Agents

Haijun Chen[†,ξ], Zhengduo Yang[‡,ξ], Chunyong Ding[†], Lili Chu[‡], Yusong Zhang[‡], Kristin Terry[‡], Huiling Liu[†], Qiang Shen[‡,*] and Jia Zhou[†,*]

[†]Chemical Biology Program, Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas 77555, United States

[‡]Department of Clinical Cancer Prevention, Division of Cancer Prevention and Population Sciences, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, United States

[ξ]These authors contribute equally to this work.

*Corresponding authors:
Jia Zhou, PhD
Chemical Biology Program
Department of Pharmacology and Toxicology
University of Texas Medical Branch
Galveston, Texas 77555, United States
Email: jizhou@utmb.edu

Qiang Shen, MD, PhD
Department of Clinical Cancer Prevention
Division of Cancer Prevention and Population Sciences
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030, United States
Email: qshen@mdanderson.org

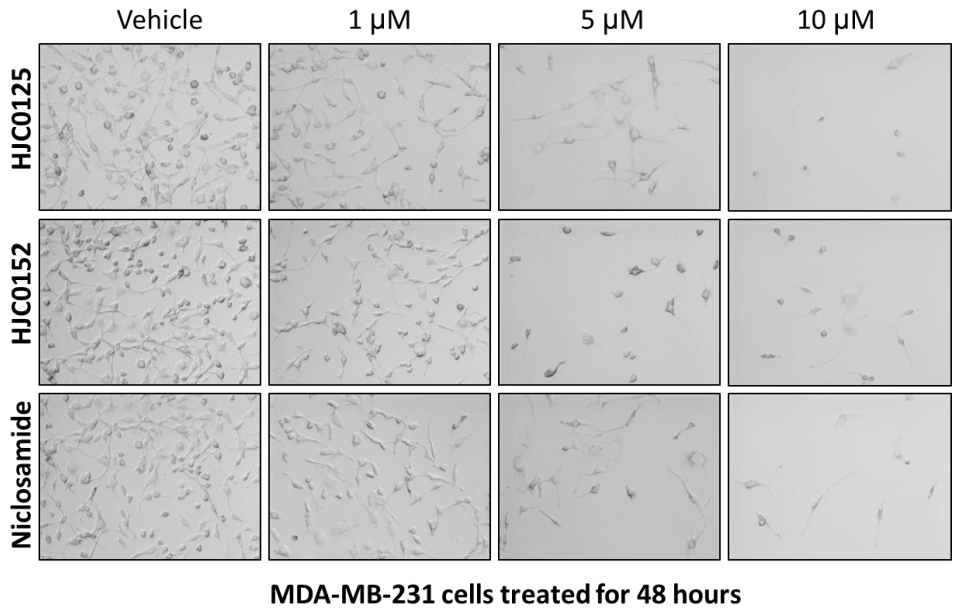

Figure S1. Effects of **10** (**HJC0125**), **11** (**HJC0152)** and niclosamide on cell growth and cellular morphological changes. Exponentially growing MDA-MB-231 breast cancer cells were incubated with compounds **10**, **11** or niclosamide for 48 h. Cell morphology was evaluated under light microscopy.

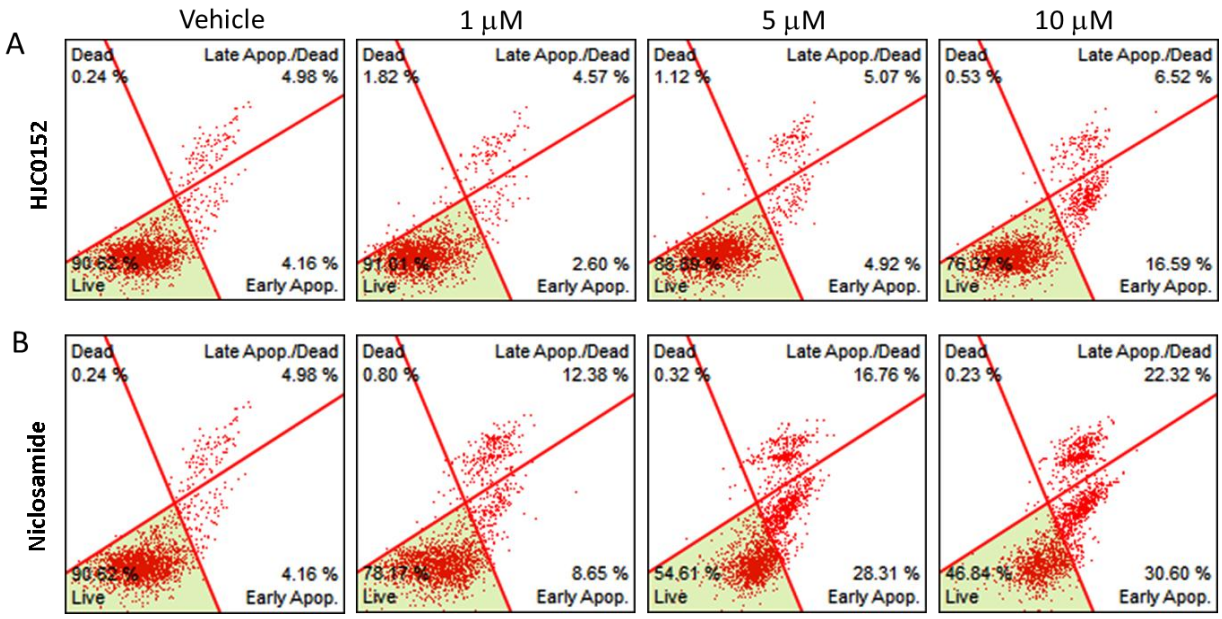

Figure S2. Induction of apoptosis on MDA-MB-231 cells by **HJC0152** (A) or niclosamide (B). Cells were not treated or treated with 1 μM, 5 μM, and 10 μM concentration of **HJC0152** or niclosamide for 48 h.

EXPERIMENTAL SECTION

General. All commercially available starting materials and solvents were reagent grade, and used without further purification. Reactions were performed under a nitrogen atmosphere in dry glassware with magnetic stirring. Preparative column chromatography was performed using silica gel 60, particle size 0.063-0.200 mm (70-230 mesh, flash). Analytical TLC was carried out employing silica gel 60 F254 plates (Merck, Darmstadt). Visualization of the developed chromatograms was performed with detection by UV (254 nm). NMR spectra were recorded on a Brucker-600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with TMS as an internal reference. Chemical shifts were expressed in ppm, and *J* values were given in Hz. High-resolution mass spectra (HRMS) were obtained from Thermo Fisher LTQ Orbitrap Elite mass spectrometer. Parameters include the following: Nano ESI spray voltage was 1.8 kV; Capillary temperature was 275 ^{o}C and the resolution was 60,000; Ionization was achieved by positive mode. Melting points were measured on a Thermo Scientific Electrothermal Digital Melting Point Apparatus and uncorrected. Purity of final compounds was determined by analytical HPLC, which was carried out on a Shimadzu HPLC system (model: CBM-20A LC-20AD SPD-20A UV/VIS). HPLC analysis conditions: Waters μBondapak C18 (300 × 3.9 mm); flow rate 0.5 mL/min; UV detection at 270 and 254 nm; linear gradient from 30% acetonitrile in water (0.1% TFA) to 100% acetonitrile (0.1% TFA) in 20 min followed by 30 min of the last-named solvent. All biologically evaluated compounds are > 95% pure.

2-(2-Bromoethoxy)-5-chloro-*N*-(2-chloro-4-nitrophenyl)benzamide (2). To a solution of niclosamide (654 mg, 2.0 mmol) and Ph_3P (1.05 g, 4.0 mmol) in THF (10 mL) was added 2-bromoethanol (500 mg, 4.0 mmol) and DIAD (606 mg, 3.0 mmol). The mixture was stirred at r.t. for 16 h. The reaction mixture was diluted with EtOAc (100 mL) and extracted with H_2O (40

mL). The organic layer was washed with brine (10 mL), dried over anhydrous Na_2SO_4, and concentrated to give the crude product. This residue was purified with silica gel column (hexane/EtOAc = 2/1 to 1/2) to provide **2** (650 mg, 75%) as a white solid (mp 162-163 ^oC). ^1H NMR (600 MHz, DMSO-d_6) δ 10.51 (s, 1H), 8.62 (d, 1H, J = 9.0 Hz), 8.45 (d, 1H, J = 2.4 Hz), 8.28-8.30 (m, 1H), 7.97 (d, 1H, J = 2.4 Hz), 7.68-7.70 (m, 1H), 7.40 (d, 1H, J = 9.6 Hz), 4.70 (t, 2H, J = 6.0 Hz), 3.92 (t, 2H, J = 6.0 Hz). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.2, 154.6, 143.2, 140.7, 133.8, 130.7, 125.8, 124.8, 123.8, 123.6, 122.8, 122.3, 116.4, 69.9, 30.9.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(2-fluoroethoxy)benzamide (3). Compound **3** was prepared in 86% yield by a procedure similar to that used to prepare compound **2**. The title compound was obtained as a white solid (mp 174-175 ^oC). HPLC purity 95.4% (t_R = 22.73 min). ^1H NMR (600 MHz, DMSO-d_6) δ 10.53 (s, 1H), 8.66 (d, 1H, J = 9.6 Hz), 8.43 (d, 1H, J = 3.0 Hz), 8.28-8.30 (m, 1H), 7.98 (d, 1H, J = 3.0 Hz), 7.69-7.70 (m, 1H), 7.40 (d, 1H, J = 9.0 Hz), 4.82-4.91 (m, 2H), 4.60-4.66 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.2, 155.0, 143.1, 140.7, 133.7, 130.7, 125.7, 124.8, 123.6, 123.3, 122.7, 122.0, 116.3, 81.6 (d, J = 165.6 Hz), 69.6 (d, J = 18.8 Hz). HRMS (ESI) calcd for $C_{15}H_{12}Cl_2FN_2O_4$ 373.0153 $(M + H)^+$, found 373.0146.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(1-methyl-piperidin-4-yloxy)benzamide (4). Compound **4** was prepared in 57% yield by a procedure similar to that used to prepare compound **2**. The title compound was obtained as a pale yellow solid (mp 161-162 ^oC). HPLC purity 97.4% (t_R = 16.69 min). ^1H NMR (600 MHz, DMSO-d_6) δ 10.33 (s, 1H), 8.63 (d, 1H, J = 9.0 Hz), 8.44 (d, 1H, J = 2.4 Hz), 8.30 (d, 1H, J = 9.0 Hz), 7.91 (s, 1H), 7.64 (d, 1H, J = 9.0 Hz), 7.44 (d, 1H, J = 9.0 Hz), 4.69-4.71 (m, 1H), 2.67-2.69 (m, 2H), 2.16 (s, 3H), 2.14-2.16 (m, 2H), 2.03-2.05 (m, 2H), 1.79-1.81 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.6, 154.0, 143.1, 140.5, 133.5,

130.6, 125.2, 124.9, 123.7, 123.6, 123.3, 122.2, 117.4, 75.8, 52.6, 45.4, 30.5. HRMS (ESI) calcd for $C_{19}H_{20}Cl_2N_3O_4$ 424.0825 $(M + H)^+$, found 424.0828.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(2-dimethylaminoethoxy)benzamide (5). Compound **5** was prepared in 50% yield by a procedure similar to that used to prepare compound **2**. The title compound was obtained as a pale yellow solid (mp 117-118 ^oC). HPLC purity 99.6% (t_R = 15.59 min). ^1H NMR (600 MHz, $CDCl_3$) δ 10.59 (s, 1H), 8.76 (d, 1H, J = 9.0 Hz), 8.33 (d, 1H, J = 2.4 Hz), 8.22 (d, 1H, J = 2.4 Hz), 8.20 (d, 1H, J = 9.0 Hz), 7.48 (dd, 1H, J = 2.4, 8.4 Hz), 7.06 (d, 1H, J = 9.0 Hz), 4.36 (t, 2H, J = 6.6 Hz), 2.80 (t, 2H, J = 6.0 Hz), 2.26 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.8, 155.5, 143.4, 141.4, 133.9, 132.6, 127.6, 125.0, 123.8, 123.5, 123.0, 122.3, 115.1, 68.6, 57.8, 45.8. HRMS (ESI) calcd for $C_{17}H_{18}Cl_2N_3O_4$ 398.0669 $(M + H)^+$, found 398.0670.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(2-morpholin-4-yl-ethoxy)benzamide (6). Compound **6** was prepared in 58% yield by a procedure similar to that used to prepare compound **2**. The title compound was obtained as a pale yellow solid (mp 152-153 ^oC). HPLC purity 98.8% (t_R = 15.64 min). ^1H NMR (600 MHz, $CDCl_3$) δ 10.62 (s, 1H), 8.75 (d, 1H, J = 9.6 Hz), 8.31 (s, 1H), 8.20 (s, 1H), 8.19 (d, 1H, J = 9.6 Hz), 7.47 (d, 1H, J = 9.0 Hz), 7.05 (d, 1H, J = 9.0 Hz), 4.40 (t, 2H, J = 6.0 Hz), 3.39-3.41 (m, 4H), 2.84 (d, 2H, J = 6.0 Hz), 2.43-2.45 (m, 4H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.5, 155.2, 143.2, 141.1, 133.8, 132.4, 127.6, 124.7, 123.5, 123.4, 122.8, 122.0, 115.2, 67.5, 66.7, 56.8, 53.7. HRMS (ESI) calcd for $C_{19}H_{20}Cl_2N_3O_5$ 440.0775 $(M + H)^+$, found 440.0781.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(2-piperidin-1-yl-ethoxy)benzamide (7). To a solution of **2** (108 mg, 0.25 mmol), KI (75 mg, 0.45 mmol) and K_2CO_3 (69 mg, 0.5 mmol) in acetone (5 mL) was added piperidine (85 mg, 1.0 mmol) at 0 ^oC. The mixture was stirred at 75

^{o}C for 18 h. The solution was diluted with EtOAc (100 mL), washed with 0.1 N HCl (aq.) (10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na_2SO_4, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/1 to 1/3) to give the desired product **7** (100 mg, 91%) as a pale yellow solid (mp 134-135 ^{o}C). HPLC purity 99.2% (t_R = 16.84 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.80 (s, 1H), 8.54 (d, 1H, J = 9.0 Hz), 8.45 (s, 1H), 8.28-8.30 (m, 1H), 7.97 (s, 1H), 7.66-7.68 (m, 1H), 7.42 (d, 1H, J = 8.4 Hz), 4.44-4.46 (m, 2H), 2.68-2.70 (m, 2H), 2.30-2.32 (m, 4H), 1.22-1.24 (m, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.1, 155.6, 143.3, 140.9, 133.7, 130.5, 125.5, 124.7, 124.5, 123.5, 123.0, 122.6, 117.2, 67.6, 56.6, 53.9, 25.3, 23.7. HRMS (ESI) calcd for $C_{20}H_{22}Cl_2N_3O_4$ 438.0982 $(M + H)^+$, found 438.0985.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-[2-(4-methyl-piperazin-1-yl)-ethoxy]benzamide (8). Compound **8** was prepared in 55% yield by a procedure similar to that used to prepare compound **7**. The title compound was obtained as a pale yellow solid (mp 149-150 ^{o}C). HPLC purity 98.6% (t_R = 15.37 min). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.66 (s, 1H), 8.75 (d, 1H, J = 9.0 Hz), 8.33 (d, 1H, J = 3.0 Hz), 8.22 (d, 1H, J = 2.4 Hz), 8.20-8.21 (m, 1H), 7.47-7.49 (m, 1H), 7.06 (d, 1H, J = 8.4 Hz), 4.39 (t, 2H, J = 6.0 Hz), 2.85 (t, 2H, J = 6.6 Hz), 2.21-2.50 (m, 8H), 2.14 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.7, 155.5, 143.4, 141.4, 133.9, 132.5, 127.7, 125.0, 124.0, 123.5, 123.0, 122.4, 115.5, 68.1, 56.5, 55.0, 53.4, 46.1. HRMS (ESI) calcd for $C_{20}H_{23}Cl_2N_4O_4$ 453.1091 $(M + H)^+$, found 453.1087.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(2-piperazin-1-yl-ethoxy)benzamide (9). To a solution of niclosamide (200 mg, 0.61 mmol) and Ph_3P (288 mg, 1.1 mmol) in THF (5 mL) was added 4-(2-hydroxy-ethyl)-piperazine-1-carboxylic acid *tert*-butyl ester (253 mg, 1.1 mmol) in THF (5 mL) and DIAD (222 mg, 1.1 mmol). The mixture was stirred at r.t. for 2 h, and was then

concentrated to give the crude product. This residue was purified with silica gel column (EtOAc/hexane = 3/1) to afford 300 mg of the intermediate as a white solid. To the solution of the intermediate (300 mg) in CH_2Cl_2 (10 mL) was added TFA (3 mL) at 0 ^oC. The mixture was stirred at r.t. for 3 h, and was then concentrated. The residue was partitioned between EtOAc (250 mL) and 1 N $NaHCO_3$ (aq., 10 mL). The organic layer was washed with H_2O (10 mL) and dried over Na_2SO_4. The organic layer was concentrated. The residue was washed with EtOAc (10 mL) and then the solid was filtered to give **9** (200 mg, 74%, two steps) as a pale yellow solid (mp 247-248 ^oC). HPLC purity 98.6% (t_R = 13.77 min). ^1H NMR (600 MHz, DMSO-d_6) δ 10.48 (s, 1H), 9.70-9.80 (bs, 1H), 8.44 (d, 2H, J = 7.2 Hz), 8.30 (d, 1H, J = 8.4 Hz), 7.85 (s, 1H), 7.68 (d, 1H, J = 9.0 Hz), 7.39 (d, 1H, J = 8.4 Hz), 4.65-4.68 (m, 2H), 3.39-3.65 (m, 10H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.2, 154.1, 143.9, 140.9, 132.9, 130.1, 125.9, 125.7, 124.9, 124.8, 124.4, 123.4, 115.9, 64.0, 58.9, 54.3, 48.5. HRMS (ESI) calcd for $C_{19}H_{21}Cl_2N_4O_4$ 439.0934 $(M + H)^+$, found 439.0911.

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-(piperidin-4-yloxy)benzamide (10). Compound **10** was prepared in 34% yield (two steps) by a procedure similar to that used to prepare compound **9**. The title compound was obtained as a pale yellow solid (mp 239-240 ^oC). HPLC purity 98.6% (t_R = 16.62 min). ^1H NMR (600 MHz, CD_3OD) δ 8.71 (d, 1H, J = 9.6 Hz), 8.39 (s, 1H), 8.24 (d, 1H, J = 9.6 Hz), 7.96 (s, 1H), 7.55 (d, 1H, J = 9.0 Hz), 7.33 (d, 1H, J = 9.0 Hz), 4.91-4.94 (m, 1H), 3.40-3.43 (m, 2H), 3.13-3.18 (m, 2H), 2.32-2.35 (m, 2H), 2.04-2.10 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.0, 153.4, 143.4, 140.6, 133.1, 130.4, 125.3, 124.9, 124.6, 124.0, 123.6, 122.8, 116.8, 72.5, 41.4, 27.8. HRMS (ESI) calcd for $C_{18}H_{18}Cl_2N_3O_4$ 410.0669 $(M + H)^+$, found 410.0671.

2-(2-Amino-ethoxy)-5-chloro-*N*-(2-chloro-4-nitrophenyl)benzamide (11). Compound **11** was prepared in 55% yield (two steps) by a procedure similar to that used to prepare compound **9**. The title compound was obtained as a pale yellow solid (mp 157-158 ^{o}C). HPLC purity 97.0% (t_R = 15.26 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.40 (s, 1H), 8.57 (d, 1H, J = 9.6 Hz), 8.55-8.56 (bs, 2H), 8.39 (d, 1H, J = 2.4 Hz), 8.24-8.26 (m, 1H), 7.90 (d, 1H, J = 3.0 Hz), 7.63-7.65 (m, 1H), 7.38 (d, 1H, J = 9.0 Hz), 4.60 (t, 2H, J = 5.4 Hz), 3.27 (t, 2H, J = 5.4 Hz). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.7, 154.5, 143.4, 141.1, 133.7, 130.9, 125.7, 124.9, 124.6, 123.6, 123.6, 122.8, 115.8, 66.4, 38.1. HRMS (ESI) calcd for $C_{15}H_{14}Cl_2N_3O_4$ 370.0356 $(M + H)^+$, found 370.0357.

2-(3-Aminopropoxy)-5-chloro-*N*-(2-chloro-4-nitrophenyl)benzamide (12). Compound **12** was prepared in 52% yield (two steps) by a procedure similar to that used to prepare compound **9**. The title compound was obtained as a pale yellow solid (mp 206-207 ^{o}C). HPLC purity 95.6% (t_R = 15.74 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.40 (bs, 1H), 8.61 (d, 1H, J = 9.6 Hz), 8.45 (d, 1H, J = 1.8 Hz), 8.31 (d, 1H, J = 9.0 Hz), 7.93 (d, 1H, J = 2.4 Hz), 7.69-7.83 (m, 2H), 7.70 (d, 1H, J = 9.0 Hz), 7.36 (d, 1H, J = 8.4 Hz), 4.42 (t, 2H, J = 6.0 Hz), 2.96 (t, 2H, J = 7.2 Hz), 2.08-2.13 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.7, 154.9, 143.2, 140.7, 133.4, 130.4, 125.2, 124.9, 123.8, 123.6, 123.2, 122.5, 115.7, 66.8, 36.1, 26.5. HRMS (ESI) calcd for $C_{16}H_{16}Cl_2N_3O_4$ 384.0512 $(M + H)^+$, found 384.0514.

2-(4-Aminobutoxy)-5-chloro-*N*-(2-chloro-4-nitrophenyl)benzamide (13). Compound **13** was prepared in 46% yield (two steps) by a procedure similar to that used to prepare compound **9**. The title compound was obtained as a white solid (mp 186-187 ^{o}C). HPLC purity 99.5% (t_R = 16.59 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.70 (d, 1H, J = 9.6 Hz), 8.46 (d, 1H, J = 2.4 Hz), 8.30-8.32 (m, 1H), 7.97 (d, 1H, J = 3.0 Hz), 7.68-7.70 (m, 1H), 7.40 (d, 1H, J = 9.0

Hz), 4.40 (t, 2H, *J* = 6.6 Hz), 2.81 (t, 2H, *J* = 7.2 Hz), 1.87-1.90 (m, 2H), 1.64-1.67 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.4, 155.2, 143.0, 140.8, 133.7, 130.6, 125.2, 124.8, 123.8, 123.2, 122.6, 121.9, 116.0, 69.3, 38.6, 25.2, 24.0. HRMS (ESI) calcd for $C_{17}H_{18}Cl_2N_3O_4$ 398.0669 $(M + H)^+$, found 398.0680.

2-(5-Aminopentyloxy)-5-chloro-*N*-(2-chloro-4-nitrophenyl)benzamide (14). Compound **14** was prepared in 73% yield (two steps) by a procedure similar to that used to prepare compound **9**. The title compound was obtained as a white solid (mp 190-191 ^{o}C). HPLC purity 97.5% (t_R = 17.57 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.54 (s, 1H), 8.68 (d, 1H, *J* = 9.6 Hz), 8.43 (d, 1H, *J* = 2.4 Hz), 8.29-8.31 (m, 1H), 7.97 (d, 1H, *J* = 2.4 Hz), 7.66-7.68 (m, 3H), 7.38 (d, 1H, *J* = 9.0 Hz), 4.35 (t, 2H, *J* = 6.6 Hz), 2.74-2.76 (m, 2H), 1.84-1.89 (m, 2H), 1.55-1.60 (m, 2H), 1.42-1.55 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.3, 155.3, 143.0, 140.7, 133.6, 130.5, 125.1, 124.7, 123.6, 123.0, 122.5, 121.8, 115.9, 69.8, 38.6, 27.7, 26.5, 22.1. HRMS (ESI) calcd for $C_{18}H_{20}Cl_2N_3O_4$ 412.0825 $(M + H)^+$, found 412.0824.

2-[2-(2-Aminoethoxy)-ethoxy]-5-chloro-*N*-(2-chloro-4-nitrophenyl)benzamide (15). Compound **15** was prepared in 81% yield (two steps) by a procedure similar to that used to prepare compound **9**. The title compound was obtained as a pale yellow solid (mp 177-178 ^{o}C). HPLC purity 99.3% (t_R = 16.19 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.59 (s, 1H), 8.63 (d, 1H, *J* = 9.0 Hz), 8.43 (s, 1H), 8.29 (d, 1H, *J* = 7.2 Hz), 7.97 (s, 1H), 7.78-7.83 (m, 2H), 7.68 (d, 1H, *J* = 8.4 Hz), 7.42 (d, 1H, *J* = 8.4 Hz), 4.48-4.52 (m, 2H), 3.86-3.89 (m, 2H), 3.58-3.62 (m, 2H), 2.86-2.90 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 162.2, 155.4, 143.0, 140.7, 133.8, 130.6, 125.5, 124.7, 123.6, 123.4, 122.5, 122.0, 116.4, 69.5, 68.2, 66.7, 38.3. HRMS (ESI) calcd for $C_{17}H_{18}Cl_2N_3O_5$ 414.0618 $(M + H)^+$, found 414.0632.

4-[4-Chloro-2-(2-chloro-4-nitrophenylcarbamoyl)phenoxy]piperidine-1-carboxylic

acid *tert*-butyl ester (17). To a solution of niclosamide (200 mg, 0.6 mmol) and Ph_3P (288 mg, 1.1 mmol) in THF (10 mL) was added *tert*-butyl 4-hydroxypiperidine-1-carboxylate (222 mg, 1.1 mmol) and DIAD (222 mg, 1.1 mmol). The reaction mixture was stirred at r.t. for 4 h, and then partitioned between EtOAc (50 mL) and H_2O (20 mL). The organic layer was washed with brine (10 mL), dried over anhydrous Na_2SO_4, and concentrated to give the crude product. This residue was purified with silica gel column (hexane/EtOAc = 3/1) to afford **17** (180 mg, 58%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.17 (s, 1H), 8.85 (d, 1H, J = 9.6 Hz), 8.32 (s, 1H), 8.20 (d, 1H, J = 6.0 Hz), 8.17 (s, 1H), 7.46 (d, 1H, J = 8.4 Hz), 7.03 (d, 1H, J = 6.0 Hz), 4.61-4.63 (m, 1H), 3.99-3.41 (m, 2H), 2.98-3.02 (m, 2H), 2.10-2.12 (m, 2H), 1.81-1.83 (m, 2H), 1.45 (s, 9H).

5-Chloro-*N*-(2-chloro-4-methanesulfonylaminophenyl)-2-(piperidin-4-yloxy)-benzamide (19). To a solution of **17** (200 mg, 0.39 mmol) in 5 mL of MeOH was added 1 mL of saturated NH_4Cl (aq.). Zinc dust (254 mg, 15.3 mmol) was added into the solution at 0 ^{o}C. The reaction was stirred at r.t. for 16 h. TLC indicated that the starting material disappeared. 100 mL of MeOH was added to the solution. The Zinc solid was filtered, and the filtrate was concentrated under vacuum to give 188 mg of **18** as a yellow solid without further purification. To the solution of **18** (100 mg, 0.21 mmol) in 10 mL of acetone was added Et_3N (42 mg, 0.42 mmol). $MeSO_2Cl$ (37 mg, 0.32 mmol) was added dropwise at 0 ^{o}C. The resulting mixture was stirred at r.t. for 4 h, and then concentrated. The residue was diluted with EtOAc (50 mL) and washed with water (20 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The solution was concentrated to give 110 mg of the crude intermediate. To the solution of the intermediate (110 mg) in CH_2Cl_2 (3 mL) was added TFA (1 mL) at 0 ^{o}C. The mixture was stirred at r.t. for 3 h, and then concentrated. The residue was partitioned between EtOAc (25 mL) and 1

N $NaHCO_3$ (aq., 5 mL). The organic layer was washed with H_2O (5 mL) and dried over Na_2SO_4. The organic layer was concentrated, and the residue was purified with silica gel column (hexane/EtOAc = 1/1) to afford **19** (30 mg, 31%, three steps) as a pale yellow solid (170-171 ^oC). HPLC purity 98.8% (t_R = 12.90 min). ^1H NMR (600 MHz, acetone-d_6) δ 8.49 (d, 1H, J = 9.0 Hz), 8.12 (d, 1H, J = 3.6 Hz), 7.57-7.59 (m, 1H), 7.54 (d, 1H, J = 3.0 Hz), 7.48 (d, 1H, J = 9.0 Hz), 7.36-7.38 (m, 1H), 4.98-5.01 (m, 1H), 3.35-3.33 (m, 2H), 3.07 (s, 3H), 3.02-3.06 (m, 2H), 2.35-2.37 (m, 2H), 2.04-2.11 (m, 2H). ^{13}C NMR (150 MHz, acetone-d_6) δ 162.6, 155.0, 136.2, 133.8, 132.6, 132.3, 126.9, 125.2, 125.0, 124.6, 121.9, 120.5, 117.6, 76.4, 43.8, 39.5, 31.3, 30.6. HRMS (ESI) calcd for $C_{19}H_{22}Cl_2N_3O_4S$ 458.0703 $(M + H)^+$, found 458.0704.

***N*-(4-Acetylamino-2-chlorophenyl)-5-chloro-2-(piperidin-4-yloxy)benzamide (20)**. Compound **20** was prepared in 66% yield (three steps) by a procedure similar to that used to prepare compound **19**. The title compound was obtained as a pale yellow solid (mp 66-67 ^oC). HPLC purity 98.3% (t_R = 12.31 min). ^1H NMR (600 MHz, acetone-d_6) δ 8.37-8.40 (m, 1H), 8.07-8.08 (m, 2H), 7.55-7.56 (m, 1H), 7.45-7.48 (m, 2H), 5.04-5.06 (m, 1H), 3.45-3.49 (m, 2H), 3.16-3.20 (m, 2H), 2.41-2.45 (m, 2H), 2.14-2.20 (m, 2H), 1.95 (s, 3H). ^{13}C NMR (150 MHz, acetone-d_6) δ 169.0, 162.5, 154.8, 137.5, 133.7, 132.2, 131.1, 127.0, 125.5, 124.5, 124.1, 120.2, 118.7, 117.4, 75.1, 43.1, 30.6, 24.2. HRMS (ESI) calcd for $C_{20}H_{22}Cl_2N_3O_3$ 422.1033 $(M + H)^+$, found 422.1034.

***In Vitro* Determination of Effects of Synthesized Compounds on Cancer Cell Proliferation.** Cancer cells (breast cancer cell lines MCF-7 and MDA-MB-231, pancreatic cancer cell lines AsPC-1 and Panc-1) were seeded in 96-well plates at a density of 2×10^3 cells/well and treated with DMSO, 0.01, 0.1, 1, 5, 10, and 100 μM of individual STAT3 inhibitors for 72 h. Proliferation was measured by treating cells with the 3-(4,5-dimethylthiazol-

2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) in a CellTiter 96t AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). Absorbance of all wells was determined by measuring OD at 550 nm after 1 h incubation at 37 ^{o}C on a 96-well iMark^{TM} Microplate Absorbance Reader (BioRad, Hercules, CA). Each individual compound was tested in quadruplicate wells for each concentration.

Determination of Water Solubility. Water solubility for **10** (as HCl salt) or **11** (as HCl salt) was determined by HPLC analysis according to a previously published protocol.[1] Solid samples of **10** or **11** (2-4 mg) were weighed and added to 1 mL water. The suspensions were shaken for 24 h at 25 ^{o}C and then centrifuged, and the supernatants were filtered. Aliquots (10 μL) of the supernatants were injected into the HPLC system equipped with a C18 reverse-phase column under the same condition which was described in the general experimental section. One point calibration[2] was done by injecting 10 μL aliquots of the corresponding buffer solutions of **10** (free base) or **11** (free base) with known concentrations.

Morphological Changes. MDA-MB-231 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and grown in RPMI 1640 (Cellgro, VA, USA) overnight at 37 ^{o}C in a humidified atmosphere of 95% air and 5% CO_2. The cells were then treated with vehicle and individual compounds. Growth media was removed 48 h after treatment and replaced with 1 × PBS. Images were taken using Olympus BX41 microscope (Olympus, Melville, USA).

Transient Transfection and Dual Luciferase Reporter Assays. MDA-MB-231 cells were pre-treated with niclosamide or **11** (**HJC0152**) at 10 μM and 20 μM concentrations for 24 h. Then the cells were trypsinized and seeded in 24-well plate at a density of 5×10^4 cells/well in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin. Transient transfections were performed 4 h after plating, using the method described previously.[3] Total

amount of DNA for transfections was 0.5 μg/well, including pSTAT3-Luc (95%, obtained from Panomics, Cat# LR0077) and internal control vector renilla (5%, from Promega, Madison, WI, USA). 5 h after transfection, the cells were treated with niclosamide or compound **11** (**HJC0152**) for 24 h, then reporter activity was evaluated using dual luciferase reporter assay kit (Promega, Madison, WI, USA) on an Omega^{TM} Microplate Luminometer (BMG LABTECH Inc., NC, USA). Relative luciferase units were the ratio of the absolute activity of firefly luciferase to that of renilla luciferase. Experiments were conducted with triplicates and results are representatives of at least 3 independent experiments.

Western Blot Analysis. Protein levels were determined by Western blot using the previously described methods.[3] Total cell lysates were prepared from MDA-MB-231 cells. Protein concentrations were measured using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Equal amounts of total cellular protein extract (40 μg) were resuspended in denaturing sample loading buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 0.1% bromophenol blue, and 20% glycerol), separated by electrophoresis on a 10% polyacylamide SDS-PAGE gel and then electrophoretically transferred to a nitrocellulose membrane (Thermo Scientific, IL, USA) at 100 Volts for 1 h at 4 ^{o}C. The membrane was then incubated in a blocking solution containing 5% non-fat milk and 1% Tween 20 in TBS for 1 h. The membrane was then incubated with antibodies specific for: phospho-STAT3-PY705 (1:3000, Epitomics, #2236-1), STAT3 (1:2000, Cell Signaling, #4904), Caspase-3-active (1:2000, Epitomics, #1476-1), Cyclin D1 (1:10000, Epitomics, #2261-1) and β-actin (1:10000, Sigma, clone AC-15). An anti-rabbit or anti-mouse secondary antibody (Amersham, Piscataway, NJ) was used at 1:4000 dilution. The Western blotted bands were visualized using ECL procedure according to the manufacturer's instructions (Amersham).

Cell Apoptosis Assay. Breast cancer MDA-MB-231 cells were incubated in 6-well plates (2.5×10^5/well). Cells were then treated with DMSO, niclosamide or compound **11** (**HJC0152**) at different concentrations for 48 h, and then both adherent and floating cells were collected, washed once with PBS. Resuspended cells were incubated with 100 μL PBS containing 1% BSA and 100 μL Annexin V and dead cell detection reagent at room temperature for 20 min. Apoptosis was measured immediately using the Muse Cell Analyzer with the Muse™ Apoptosis Kit (Catalog No. MCH100105).

***In Vivo* Antitumor Activity Assays.** All procedures including mice and *in vivo* experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of UT M.D. Anderson Cancer Center (MDACC). Fifty-four female nude mice were obtained from MDACC and were used for orthotopic tumor studies at 4 to 6 weeks of age. The mice were maintained in a barrier unit with 12 h light-dark switch. Freshly harvested MDA-MB-231 cells (2.5×10^6 cells per mouse, resuspended in 100 μL PBS) were injected into the 3rd mammary fat pad of the mice, and then randomly assigned into 8 groups (5-10 mice per group). For the intraperitoneal treatment experiment, the mice were treated daily with 2.5 mg/kg compound **11** (**HJC0152)** (Group A), 7.5 mg/kg **HJC0152** (Group B), 12.5 mg/kg niclosamide (Group C), or vehicle (Group D) when the tumor volume reached 200 mm^3. Similarly, for the oral gavage experiment, the mice were given 25 mg/kg **HJC0152** (Group E), 75 mg/kg **HJC0152** (Group F), 75 mg/kg niclosamide (Group G), or vehicle (Group H) five days per week when the tumor volume reached 200 mm^3. All drugs were dissolved in 50% DMSO with 50% polyethylene glycol for *in vivo* administration. Body weights and tumors volume were measured daily and tumor volume was calculated according to the formula $V = 0.5 \times L \times W^2$, where L = length (mm) and W = width (mm).

Statistical Analysis. Statistical significance was determined using student t-test in cell cycle analysis. * represents a *p* value less than 0.05.

REFERENCES

1. Vogel, G. H. Determination of solubility by hyphenated HPLC methods. *In Drug Discovery and Evaluation: Safety and Pharmacokinetics Assay*; Springer: New York, **2006**; pp 400-402.

2. Kiselev, E.; DeGuire, S.; Morrell, A.; Agama, K.; Dexheimer, T. S.; Pommier, Y.; Cushman, M. 7-azaindenoisoquinolines as topoisomerase I inhibitors and potential anticancer agents. *J. Med. Chem.* **2011**, *54*, 6106-6116.

3. Shen, Q.; Uray, I. P.; Li, Y.; Krisko, T. I.; Strecker, T. E.; Kim, H. T.; Brown, P. H. The AP-1 transcription factor regulates breast cancer cell growth via cyclins and E2F factors. *Oncogene* **2008**, *27*, 366-377.