Supplementary Information

Cytotoxic potential of di-spirooxindolo/acenaphthoquino andrographolide derivatives against MCF7 cell lines

Debanjana Chakraborty[a], Arindam Maity[a], Chetan K. Jain[b,c], Abhijit Hazra[a], Yogesh P. Bharitkar[a], Tarun Jha[d], Hemanta K. Majumder[b], Susanta Roychoudhury[c], Nirup B. Mondal[a*]

[a]Department of Chemistry, [b]Molecular Parasitology Laboratory, [c]Cancer Biology and Infllamatory Disorder Division, Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, 4 Raja S C Mullick Road, Jadavpur, Kolkata 700032, West Bengal, India.

[d]Department of Pharmaceutical Technology, Division of Medicinal and Pharmaceutical Chemistry, PO Box No. 17020, Jadavpur University, Kolkata-700 032, West Bengal, India

Experimental section

Instrumentation and chemicals

All the compounds were synthesized in one-pot sequences using a mono mode Discover microwave reactor (CEM Corp., Matthews, NC, U.S.A). Melting points were determined in capillaries and are uncorrected. IR spectra were recorded as KBr pellets using a JASCO 410 FTIR spectrometer. The NMR spectra were recorded in DMSO-d_6 /$CDCl_3$ with TMS as internal standard using a Bruker 600 DPX spectrometer operating at 600 MHz for ^1H and 150 MHz for ^{13}C. ESI mass spectra (positive mode) were obtained on a Q-TOF micro TM mass spectrometer. Isatins, acenaphthoquinone, α-amino acids and other chemicals were purchased from Alfa-Aesar Company. All other solvents and chromatographic absorbents were procured from E. Merck (Germany) and SRL (India) Ltd. unless otherwise indicated.

Biology

Cell lines (CHO, HepG2, HeLa, A-431, MCF-7, MDCK and Caco-2) were obtained from National Centre for Cell Sciences, Pune, India. MEM-alpha, FBS (fetal bovine serum), penicillin and streptomycin were purchased from Gibco, India. Cell culture flasks, plates, and

tips were obtained from Tarsons, Kolkata, India. DMEM, other components of media, and all other chemicals were purchased from Sigma, St. Louis, USA.

Cell culture and cell viability assays

HeLa cells were plated (in 96 well plates) at 6000 cells/well/180 µl media. For other cell lines it was 4000 cells/well/180 µl media. Cell seeding density was optimized so that the wells without any inhibitor could make up to 90% confluency at the end of the incubation period/experiment. The plates containing cells were placed in a 37 °C incubator with 5% CO_2 and 95% relative humidity for 24 h. The media was then aspirated off and replaced with 180 µl of fresh media. Test compounds were dissolved at a concentration of 5 mM (DMSO), and further serial half dilutions were made in DMSO to reach a concentration of 0.078 mM. These sub stocks were further 10-fold diluted in respective growth media. Then 20 µl of growth media containing test compounds was added (n = 2) in a 96-well test plate to produce final working concentration of 50 to 0.78 µM (DMSO: 1%). Each plate had cell control, vehicle control, and media control and standard inhibitor Doxorubicin at 10µM.

MDCK cells were maintained in growth media containing MEM-alpha supplemented with 10% FBS and penicillin–streptomycin (100 U/ml for each). Other cell lines were maintained in DMEM media containing 10% FBS and 40 µg/ml gentamycin. The plates were placed back into the incubator for 72 h. An aliquot (20 µl) of MTT solution (5 mg/ml in PBS of pH 7.2) was added in each well, and the plates were further incubated for 4 h. The plates were centrifuged (2500 rpm, 10 min), media was flicked off, and the formazan crystals were dissolved in 150 µl of DMSO. Absorbance was measured at 510 nm using Spectramax M5 (Molecular Devices, U.S.A.). Cell death at each concentration was determined based on OD difference of the test well from that of wells of vehicle control. If the highest test concentration (50 µM) showed less than 50% cell death, then IC_{50} is reported as >50 µM; otherwise it was calculated using Graph Pad Prism 5.0 software. The MTT assay experiment

was performed in duplicate wells each day, and on three separate days.[1,2] From the IC_{50} values we could select five derivatives of andrographolide (**5b, 5c, 5i, 5g** and **8**) which are more active in MCF-7 cell lines.

Assessment of cell morphology

Cells (3×10^4/well) were grown in 6-well TC plates and treated with or without **5b, 5c, 5i, 5g** and **8** at IC_{50} concentration for 24 h. Morphological changes were observed with an inverted phase contrast microscope (Model: OLYMPUS IX70, Olympus Optical Co. Ltd., Shibuya-ku, Tokyo, Japan) and photographs were taken with the help of a digital camera (Olympus Inc., Japan).

Apoptosis Assay

Apoptosis assay was done by using an annexin V-FITC apoptosis detection kit (Calbiochem, Germany). Cells treated with or without selected andrographolide derivatives were stained with PI and annexin V-FITC according to manufacturer's instructions. Briefly, MCF7 cells (1×10^6/ml) were incubated with or without **5b** (IC_{50}: 10.9±0.4 μM), **5c** (IC_{50}:12.7±0.1 μM), **5g** (IC_{50}:12.8±0.9 μM), **5i** (IC_{50}:13.7±1.7 μM) and **8** (IC_{50}:8.9±1.6 μM) for 24 h and 48 h at 37°C, 5% CO_2. Following the treatment period, the floating cells were collected and the adherent cells were trypsinized. Both floating and adherent cells were washed in medium, centrifuged, and then resuspended in 2 ml of medium. Cells were then washed twice in PBS and resuspended in Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Annexin-V-FITC was then added according to the manufacturer's instructions and incubation was done for 15 min under dark conditions at 25 °C. PI (0.1 μg/ml) was added just prior to acquisition. Data was acquired using BD LSR Fortessa flow cytometer (Becton Dickinson, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm, and analyzed with BD FACS Diva software of the same company.

Detection of mitochondrial membrane potential

The mitochondria are attractive targets for cancer chemotherapy because their impairment renders cells non viable. The loss of mitochondrial potential is one of the indicators of apoptosis. The mitochondrial transmembrane electrochemical gradient ($\Delta\Psi m$) was therefore measured using mitochondrial potential sensor JC-1, a cell permeable, cationic and lipophilic dye. In viable cells, it freely crosses the mitochondrial membrane and forms J-aggregates which fluoresce red. In apoptotic cells, decrease in mitochondrial membrane potential prevents JC-1 from entering the mitochondria. Consequently, it remains as monomers in the cytosol that emits a predominantly green fluorescence.[3] Therefore, the ratio of J aggregates/monomers functions as an effective indicator of mitochondrial transmembrane potential and helps distinguish apoptotic cells from their healthy counterparts. Briefly, MCF7 cells (1×10^6/ml) were incubated with IC_{50} concentration of **5b**, **5c**, **5i**, **5g** or **8** for 24 and 48 h at 37°C, 5% CO_2. The cells were then washed with PBS and incubated with JC-1 (2 μM final conc.) according to manufacturer's instructions under dark conditions for 15-30 min at 37°C, 5% CO_2. Cells were acquired using FACS and analyzed using FACS Diva software.

Measurement of ROS generation

The intracellular ROS generation was measured by DCF-DA. To examine the effect of the andrographolide derivatives on generation of ROS, we used CM-H_2DCFDA, a lipid soluble, membrane permeable non-fluorescent reduced derivative of 2,7-dichlorofluorescein. The acetate groups of CM-H_2DCFDA are removed by intracellular esterase cleavage to produce the hydrophilic, non-fluorescent dye dichlorodihydrofluorescein ($DCFH_2$), which is subsequently oxidized by ROS to form the highly fluorescent product dichlorofluorescein (DCF). Thus, the fluorescence generated is directly proportional to the quantum of ROS generated.[4] The effect of IC_{50} concentrations of **5b**, **5c**, **5i**, **5g** and **8** on generation of ROS (12 and 24 h) was measured in cells (1×10^6/ml). After treatment, cells were washed with

PBS, resuspended in PBS, and then incubated with H_2DCFDA (20 μM in PBS) for 30 min at 37°C. Subsequently, cells were again washed and resuspended in PBS. DCF fluorescence was determined by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm by BD LSR Fortessa flow cytometer.

Western blot analysis

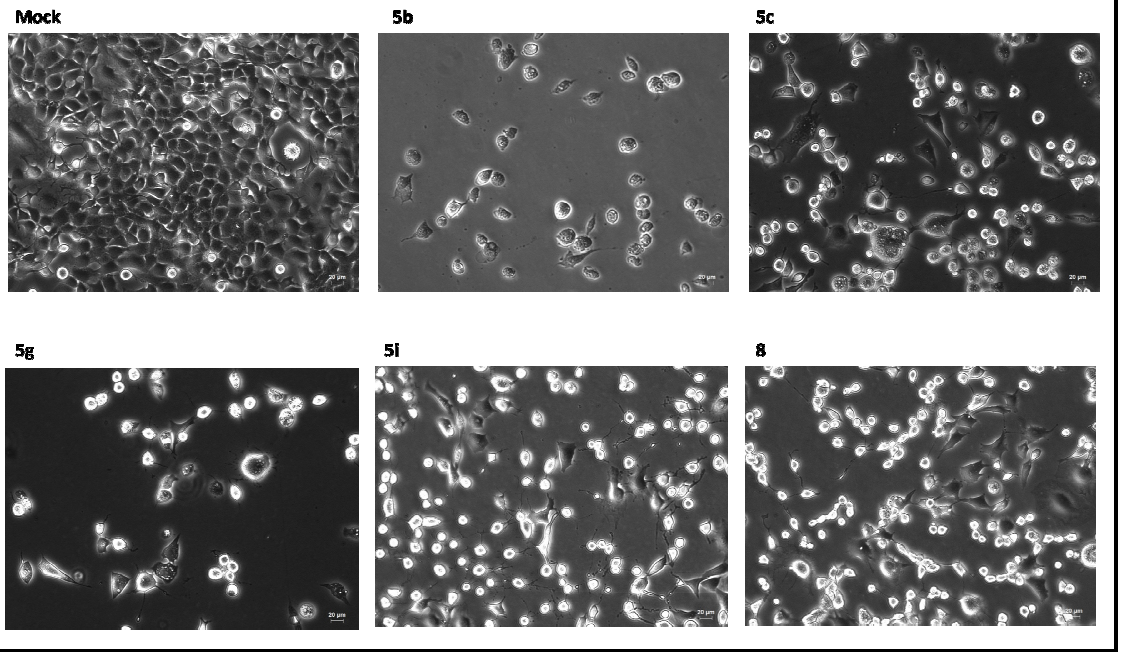
Western blot analysis was performed mainly to determine protein expression levels as mentioned earlier, with minor modifications.[5] Cells were cultured in 6 cm dishes and treated with IC_{50} concentration of **5b**, **5c**, **5i, 5g** or **8** for 24 or 48 h. These were lysed in NP-40 buffer and equal amounts of protein were electrophoresed on SDS-poly acryl amide gel. Separated proteins were then transferred on to nitrocellulose membrane and detected using antibodies available in Apoptosis Antibody Sampler Kit (Cell Signaling Technology) by western blotting.

Cell cycle analysis using flow cytometry

Sub confluent cells were treated with IC_{50} concentration of **5(b**, **c**, **i**, **g)** or **8** in culture medium for 24 and 48 h. The control and treated floating and adherent cells were collected by trypsinization and pelleted at 100 g for 5 min. Cells were harvested and washed two times with PBS and processed for cell cycle analysis. Briefly, 1×10^6 cells were re-suspended in 300 μl of cold PBS, to which 70% cold ethanol (700 μl) was added, and the cells were then incubated overnight at 4°C. After removing ethanol and washing with PBS, cells were suspended in 500 μl PBS, and incubated with 100 μg/ml RNase A for 1 h at 37°C. Then the cells were subsequently incubated with 50 μg/ml propidium iodide (PI) for another 30 min at 37°C in subdued light.[6,7] The stained cell suspension was analyzed with BD LSR Fortessa flow cytometer. The DNA content of 10,000 cells per sample was used to analyze the cell

cycle using DNA histograms. The DNA content in the cell-cycle of the analyzed cells was calculated by MODFIT 3.0 software (Verity Software House, ME, USA).

Supplementary Figure S1



Legend of S1:

Morphology of MCF-7 cells treated with 5b, 5c, 5g, 5i and 8. MCF-7 cells were treated with 5b (IC_{50}:10.9 µM), 5c (IC_{50}:12.7 µM), 5g (IC_{50}:12.8 µM), **5i** (IC_{50}:13.7 µM) and **8** (IC_{50}:8.9 µM) for 24 hours and viewed under bright-field microscopy for morphology analysis.

Reference

1. B. K. Talupula, *J. Adv. Pharm. Res.* 2011, **2**, 9−17.
2. M. Al-Qubaisi, R. Rozita, S. K. Yeap, A. R. Omar, A. M. Ali, N. B. Alitheen, *Molecules* 2011, **16**, 2944−2959.
3. D. Deb, X. Gao, H. Jiang, B. Janic, A. S. Arbab, *Biochem. Pharmacol*. 2010, **79**, 350–360.
4. A. Manna, P. Saha, A. Sarkar, D. Mukhopadhyay, A. K. Bauri, *PLoS One*, 2012, **7**, e36938.
5. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci.,* 1979, **76** (9), 4350–4354.

6. P. Carpinelli, P. Cappella, M. Losa, V. Croci, R.Bosotti, *J. Mol. Biomark. Diag.* 2011, **S2:001**. doi:10.4172/2155–9929.S2–001.
7. S. K. Mantena, D. Som, S.D. Sharma, S. K. Katiyar, *Mol. Cancer. Ther.,* 2006, **5,** 296–308.