Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supporting Information

3-Formylchromone Based Topoisomerase IIα Inhibitors: Discovery of Potent Leads

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Synthesis – General (Reference 16 and 24)

General procedure for the synthesis of 3-formylchromones (11a-e)

Variously substituted phenols were treated with acetylcholride (1.1 molar equivalents); the contents were heated for one hour. The reaction mixture was further subjected to thermal Fries rearrangement using anhydrous $AlCl_3$ (2 eq). The contents were heated for 6-7 h, and subsequently were fused with dilute HCl; reaction mixture was partitioned between ethyl acetate and water. Extracted ethyl acetate was dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under reduced pressure to obtain substituted *o*-hydroxyacetophenones. To a cooled stirred solution of various substituted *o*-hydroxyacetophenones and *N*, *N*-dimethyl-formamide was added phosphorus oxychloride drop-wise with constant stirring and the reactants were allowed to stand overnight, and then treated with ice cold water to obtain yellow crystalline solid, which was filtered and washed with diethyl ether. The crude solid was recrystallized from acetone to get variously substituted 3-formylchromones.

General procedure for the conversion of chromones to 2-anilino-3-formylchromones (12a-e)

To a clear solution of chromones (10 g) in dry benzene (150 mL) was added *N*-phenylhydroxylamine (1 molar equivalent) dissolved in dry benzene (20 mL), leading to the formation of nitrone. The obtained nitrone was further subjected to refluxing under anhydrous conditions in presence of few drops of acetic acid for 5-6 h. After the conversion, solvent was removed under reduced pressure and 2-anilino-3-formylchrome was further recrystallized from chloroform:hexane (1:2)

General procedure for the conversion of 2-anilino-3-formylchromes to 2-(N-methylanilino)-3-formylchromones (13a-e)

2-Anilino-3-formylchromes (**12a-e**, 5.0 g) were dissolved in dry acetone (150 mL) and to the solution was added K_2CO_3 (excess) and methyl iodide (2.0 molar equivalents). The contents were refluxed with stirring under anhydrous condition and the progress of reaction was monitored by TLC. After the completion of reaction (6-7 h), contents were filtered hot and residue was washed with acetone, and, solvent was removed under reduced pressure, 2-(*N*-methylanilino)-3-formylchromone were recrystallized from chloroform:hexane (1:2).

Biological assay- General

All the reagents required for the testing of new chemical entities were purchased from TopoGEN, Inc. (Columbus, OH). The testing of the compounds was performed using a commercially available Topo-II drug screening kit. All the synthesized compounds and etoposide were dissolved in DMSO at a concentration of 1 mM as a stocked solution and stored at -20 °C. Topo I and II inhibition assay were performed as described in the supplier manual with minor modifications as per the requirement.

hTopo IIα mediated DNA decatenation assay

Topo IIα mediated DNA inhibition activity for the synthesized compounds were performed as follows. Reaction mixture containing freshly prepared 5x complete reaction assay buffer (buffer A: 0.5 M Tris-HCl (pH-8), 1.50 M sodium chloride, 100 mM magnesium chloride, 5 mM dithiothreitol, 300 μg of bovine serum albumin/mL; buffer B: 20 mM ATP in water), 150 ng catenated kDNA (substrate), 100 μM drug or test compound dissolved in DMSO followed by 2-4 units of purified hTopo-IIα were incubated at 37 °C for 30 min. The reaction was then terminated

with addition of 10% SDS, followed by digestion with proteinase K. Further the reaction mixture was again incubated at 37 °C for 15 min. 20 μL of each sample were then subjected to 1% agarose gel electrophoresis in Tris-acetate- EDTA (TAE) buffer containing 0.5 μg/mL ethidium bromide and further destained with water for 20 min. The bands were analyzed under UV trans-illuminator and the decatenated kDNA products were quantified with QuantityOne (BioRad).

hTopo IIα mediated DNA relaxation assay

Supercoiled plasmid DNA (pRYG) was used as substrate in Topo II mediated DNA relaxation assay. Reaction mixture contained freshly prepared 5x complete buffer (A: 0.5MTris- HCl (pH-8), 1.50 M sodium chloride, 100 mM magnesium chloride, 5 mM dithiothreitol, 300 μg of bovine serum albumin/mL; buffer B: 20 mM ATP in water), 250 ng of supercoiled plasmid DNA (pRYG), followed by either test compound or standard drug (100 μM) and finally hTopo-IIα (2-4 units) in a total of 20 μL. Reaction mixture was then incubated at 37 °C for 30 min and stopped with addition of 10% SDS followed by digestion with proteinase K. Further the reaction mixture was again incubated at 37 °C for 15 min. Electrophoresis of the each sample was carried out in a 1% agarose gel in TAE buffer without ethidium bromide. Gel was stained with ethidium bromide (0.5 μg/mL) for 15 min and destained with water for 20 min. The bands were analyzed under UV trans-illuminator and quantification of the products was carried out as mentioned in decatenation assay.

DNA Intercalation Assay

In this assay, negatively supercoiled plasmid isolated from *E. coli* was used as a substrate. 250 ng plasmid was incubated with 1 μg/mL ethidium bromide, 100 μM standard drug (etoposide), or with the investigational compound at 37 °C for 20 min. Samples were then loaded in 1% agarose gel and electrophoresis was carried out in TAE buffer. Further the gel was stained with ethidium

bromide (0.5 μg/mL) for 15 min and destained with water for 20 min and photographed using BioRad.

MTT cell survival assay

Cell lines and cell culture

PC-3 (human prostate adenocarcinoma cells) was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in DMEM, containing 10% FBS and 1% penicillin-streptomycin. DMEM, FBS and Etoposide were obtained from Sigma Chemicals and Invitrogen. Cell cultures were maintained in flasks under standard conditions: incubation at 37 ^{o}C and 5% CO_2. All the subcultures were used prior to passage 15. Cells were routinely sub-cultured using 0.25% trypsin. For treatment, cells were cultured in the presence of increasing concentrations (1μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM) of compounds for 48 hours.

In vitro cytotoxicity measurements

All *in-vitro* experiments for cell proliferation/inhibition were performed in triplicates. For the PC-3 cell growth inhibition assay, 2.0 X 10^4 cells/well were plated in 96-well microtiter plate. After 24 hours cells were incubated in the presence and absence of increasing concentration of positive control etoposide or the test compounds (1μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM) at 37 ^{o}C and 5% CO_2. Cell proliferation was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (MTT obtained from Hi-Media). After 48 hours incubation, cells were treated with MTT solution for 4 hours in a cell culture incubator at 37 ^{o}C and 5% CO_2. MTT which is a tetrazolium salt, is converted into insoluble formazan by mitochondrial dehydrogenases in live cells. Formazan is dissolved in DMSO (Merk) and absorbance was measured at dual wavelength of 550nm and 630nm on ELISA plate

spectrophotometer (flex station).The total number (percentage) of viable cells relative to viable cells in untreated control is calculated.

Molecular modeling

Ab initio density functional theory (DFT)[36] based geometry optimization calculations of the 3-formylchrmone rotamers were performed using GAUSSIAN09 software package.[37] The gas phase geometry of all the species were fully optimized using B3LYP (Becke3, Lee, Yang, Parr) methods with 6-31+G(d,p) basis sets without any geometrical constraint. Frequencies were computed analytically for all the optimized species to characterize stationary points as minima and to estimate the zero point vibrational energies (ZPE). The calculated ZPE values (at 298.15K) were scaled by a factor of 0.9806 for the B3LYP levels.[38]

The 3D structure of the compounds (considering stable conformation revealed from quantum chemical analysis) under study were built using SYBYL 7.1 molecular modeling package[39], installed on a Silicon Graphics Fuel Work station running IRIX 6.5. Gasteiger-Hückel partial charges were applied to the atoms of the compound and Powell method with Tripos force field was used for minimization. Finally, a database of ligands was created in SYBYL. For the purpose of docking, crystal structure of the ATPase domain of hTopo-IIα[29] bound to AMPPNP (5`-adenylyl-β,γ-imidodiphosphate, a non-hydrolyzable ATP analog, PDB ID: 1ZXM, resolution 1.86 Å) was utilized. This PDB structure composed of a homodimer, each chain having bound ligand AMPPNP and Mg^{2+} ion forming distorted octahedral coordination (3-point contacts with phosphate groups, one with Asn91 and two with conserved water molecules 927 and 928) in an active site. The enzyme structure with two conserved water molecules in the active site was subjected to protein preparation by implementing the methodology reported earlier.[14]

Docking simulation was performed using FlexX (version 1.2)[30] programme incorporated in SYBYL 7.1 package. It was established in previous study [14] that score and poses obtained while keeping the two conserved water molecules in coordination with Mg^{2+} ion give more accurate results. Thus, in this work also we have employed the same protocol for molecular docking. For the protocol validation bound ligand AMPPNP and substrate ATP were docked initially, and then analyzed for their binding pose and hydrogen bonding interactions with the key residues. Afterwards, a database of ligands (**11b**, **12a**, **12b**, **12d**, **12e**, **13a** and **13b)** was subjected to docking by using FlexX multiple ligand docking mode. At the end of docking, 30 poses per ligand were generated which were sorted according to their FlexX score, reflecting binding affinity with the enzyme. Top most poses were visually inspected and analyzed for their interactions with Mg^{2+} ion and active site residues.