2. Chapter 2

Materials and methods

Logistics

Experiments were conducted in the Department of Physiology at the University of Pretoria in conjunction with the African Centre for Gene Technologies (ACGT) and the Microarray facility and the Bio-informatics and Computational Biology Unit of the University of Pretoria. Molecular modeling studies were performed on an Intel I7 920 running Ubuntu 9.04 and in collaboration with the Bio-informatics and Computational Biology Unit of the University of Pretoria. Real-time PCR was conducted at the Real-time PCR Facility at the University of Pretoria. Electron microscopy was conducted at the Electron Microscopy Unit of the University of Pretoria. Flow cytometry analysis was conducted at the department of Pharmacology of the University of Pretoria.

Ligand binding assays of the active compounds on CAII and CAIX were conducted by Dr. Katherine Sippel in the McKenna Lab at the Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Florida, USA. The effects of the compounds on microtubule dynamics were conducted by Dr. Renaud Prudent and Dr Laurence Lafanechere of the Center for Bio-Active Molecules screening (CMBA), Grenoble, France.

General *in silico* materials

Only software that is available free of charge for academic purposes was used in the present study.

Structure visualization

Various open source graphic user interfaces (GUIs) were used to generate 2-dimensional (2D) and 3-dimensional (3D) depictions of simple molecules as well as complex protein structures. These include Chimera, Chemsketch, VegaZZ, the Python Molecular Viewer (PMV) and Visual Molecular Dynamics (VMD) molecular graphics software. The University of California, San Francisco Chimera package (supported by NIH P41 RR-01081) is a highly extensible program for interactive visualization and analysis of molecular structures and related data, including density maps, supramolecular assemblies, sequence alignments, docking results, trajectories, and conformational ensembles (169). ChemSketch is a freeware drawing package from Advanced Chemistry Development, Inc. (Toronto, Canada). The software allows a user to generate Simplified Molecular Input Line Entry System (SMILES) annotations of ligands as well as 2D structures(170). The VegaZZ GUI employs an Open Graphics Library (OpenGL) interface to render high-quality interactive 3D molecules(171). PMV is part of the Autodocktools4 (ADT) package and is a python-based molecular with an OpenGL component to represent 3D molecules (172, 173). VMD is another molecular visualization program that uses OpenGL to represent proteins and other biological compounds and it also allows for animating molecules undergoing simulation(174).

Docking

Docking studies were carried out with Autodock4.0 and AutoDockTools4 (Scripps Research Institute, La Jolla, CA, USA) and Autodock Vina(172, 175). DOCK6 was also used, however, the performance of the docking software was not satisfactory as it was unable to reproduce the majority of the crystal poses of proteins used in this study (RMSD > 2, data not shown)(176). Autodock, DOCK and Autodock Vina are freely available for academic use.

Autodock 4.0 is a software suite that consists of two main components. The first component, *autogrid*, is used to calculate and generate a grid that describes the properties of a protein used as a receptor.A semi-empirical free energy force field is used to calculate the grids(172). The grid

maps that are generated take into account hydrogen bonding, electrostatic interactions, deviation from covalent geometry, internal ligand torsional constraints, and desolvation effects(172). The second component of the Autodock 4.0 suite, *autodock*, employs a Lamarckian genetic algorithm whereby random populations of a ligand are selected based on the scoring function(172). The scoring function is based on interactions between a ligand and the grid-based representation of the protein. Interactions that are calculated include dispersion/repulsion interactions, hydrogen binding, electrostatic interactions and a desolvation potential based on volume(172). The best scoring populations of the ligands undergo successive generations of conformational changes ("mutations") until the lowest binding energy or local minima is reached(172). The lowest binding energy after successive runs represents the position and energy difference of a ligand predicted to be the naturally observed.

AutoDock is one of the most widely used and successful docking software packages. For example, novel L-xylulose reductase, bcr-abl tyrosine kinase, Bcl-2 protein, Casein Kinase II and integrin alpha-v-beta3 inhibitors have been identified through VS of a library of ligands utilizing DOCK software (177-181). Novel inhibitors of protein phosphatase 2C, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, Cdc25 phosphatase inhibitors and the glucose transporter protein GLUT1 have been identified using Autodock (182-185). Autodock performed better when compared to DOCK and GOLD for differentiating between active ligands and inactive ligands based on the scoring function(186).

AutoDock Vina is a newly designed docking program from the Molecular Graphics Lab at The Scripps Research Institute and is faster and as accurate as Autodock(175). Like Autodock, Autodock Vina is a grid-based docking solution, however, the calculation of van der Waals interactions, hydrogen bonding, the deformation effect and the hydrophobic effect are calculated based on the X-Score scoring function(175, 187). The scoring function is derived using the PDBbind data set and the software implements an efficientIterated Local Search global optimizermethod to find the optimal docking pose(175). Autodock Vina is faster than Autodock and is also able to reproduce the crystal poses (RMSD $<$ 2.0) in 78% of the tested protein-ligand complexes, 29% better than Autodock 4(175).

Structure preparation

Protein structures for docking were gathered from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (188). The Reduce software package was used for adding hydrogens to the receptor Protein Data Bank molecular structure files(189). The Assisted Model Building with Energy Refinement (*AMBER*) Antechamber module (included with Chimera) was used to assign Generalized *AMBER* force field (GAFF) types and atomic partial charges to each atomic residue of the PDB structure (190, 191). Minimization of proteins was done by utilizing the Amber ff99ua forcefield as implemented in Chimera (191, 192). Three-dimensional PDB ligand atomic co-ordinates were generated from the SMILES annotations by the Online SMILES Translator service provided by the computer-aided drug design (CADD) group and the National Cancer Institute (NCI) (170). Ligand conformational searches for X-ray ligands and SMILES generated ligands were made with VEGA 2.2.0(171). Proteins were superimposed by the MatchMaker module in Chimera.

For docking with Autodock and Autodock Vina, receptors and ligands (in pdbqt format) were prepared from the mol2 files generated from VegaZZ and Chimera. The prepare_receptor4.py and prepare_ligand4.py scriptswere used, adding Gasteiger charges and merging non-polar hydrogens. These scripts are part of the Autodocktools4 package(172). For Autodock, grids were generated with *autogrid*(172).

General *in silico* methods

Receptor and ligand selection

In order to generate a library of modified estradiol ligandswith potential antimitotic and anti-carbonic anhydrase activity, several modifications were made at positions 2 and in the D-ring. Crystal structures of human CAII and a mimic of CAIX were used to evaluate the potential of the analogues to bind and inhibit them.

Human CAII is one of the most commonly studied enzymes with already 279 X-ray crystallographic structures in 2009 and a wealth of information regarding various inhibitors of CAII (214). Tuccinardi *et al.* (2007) determined via cross-docking analysis using the ChemScore GOLD docking program that the 1okn, 1kwr, 1cim, 1bnw, 1cnx, 1oq5 and 1ttm X-ray structures displayed the best RMSD average of the redocked ligands(193). The crystal structure of the wild-type CAIX was not available before 2011, therefore, various crystal structures (3dc9, 3dcs, 3dcc, 3dc3, 3dcw and 3dbu) of a mimic of the wild type was used for docking studies. The accuracy of CAIX mimic as a model of wild-type CA provides a useful tool to test isozyme-specific CA IX inhibitors(194, 195).

In order to evaluate the potential of the above-mentioned analogues to bind to the colchicine binding site, all the available crystal structures that were available at the time were chosen to be included into an ensemble docking study. These include 1sa0, 1sa1, 1z2b, 3du7, and 3e22 files from the RCSB database.Each crystal structure has two colchicine binding sites (chain A and B) and both were used for the ensemble docking to give a final total of 10 tubulin receptors.

A good docking program can accurately reproduce the crystal structure of a ligand as well as provide an accurate representation of the binding energy. 52 CAII-inhibitor complex structures from the RCSB) Protein Data Bank with known inhibition constants (K_i) was (2nng, 3bet, 1okm, 1okl, 2nns, 2h15, 2gd8, 2nn1, 1xq0, 2pov, 1z9y, 2pow, 1ttm, 2aw1, 2pou, 1eou, 2hl4, 1xpz, 1oq5, 1ze8, 2hd6, 3daz, 1kwq, 3d8w, 1kwr, 1eou, 1a42, 3d9z, 1cin, 1bnv, 1cim, 1g54, 1i90, 1i8z, 1i91, 3dd0, 1g53, 1bnw, 2f14, 1bn4, 1bn1, 1cil, 1g1d, 1bnq, 1g52, 1if8, 1bnu, 1bnt, 1bn3, 1bnn, 1bnm, 1if7). The ligands of these complexes were redocked back into 1okn, 1kwr, 1cim, 1bnw, 1cnx, 1oq5 and 1ttm receptors. The RMSD and docking energy of each ligand was evaluated in order to determine whether there was a correlation between the experimental inhibition constant and the one calculated by the docking software.

Receptor and ligand preparation

For receptor preparation, waters and other HET groups were removed. The water molecule between ASN62, ASN67 and GLN92 in the carbonic anhydrase receptors were included in docking simulations because they are present in most of the X-ray proteins analyzed (193, 195). Hydrogens were added by making use of the Reduce software. The software adds in a standardized geometry with optimization of the orientations of OH, SH, NH_3^+, Met methyls, Asn and Gln sidechain amides, and His rings (189). After adding hydrogens, the *AMBER*Antechamber module (included with Chimera) was used to assign the GAFF types and atomic partial charges to each atomic residue of the PDB structure (190, 191). After addition of hydrogens and atomic partial charges, the receptors were saved as .mol2 files and minimized utilizing the Amber ff99ua forcefield as implemented in Chimera (191, 192). 1000 steps (step size of 0.02 Angstrom) of constrained minimization of the hydrogen network was followed by 500 steps (step size of 0.02 Angstrom) of side chain atoms to allow for internal hydrogen bond formation and removal of any internal clashes. After minimization, the respective tubulin, CAII and CAIX proteins were superimposed by the MatchMaker module in Chimera and saved as .mol2 files. The ligands and the receptors were separated and saved in separate .mol2 files. The AD4_bound.dat parameters of Autodock 4.0 and the prepare_receptor4.py script were used to generate .pdbqt files of the receptors and these files were used for docking. The prepare_receptor4.py script adds Gasteiger charges and merges non-polar hydrogens (Figure 2.1). The zinc ion charge of carbonic anhydrase receptors was set to +2 as the prepare_receptor4.py script sets it to zero.

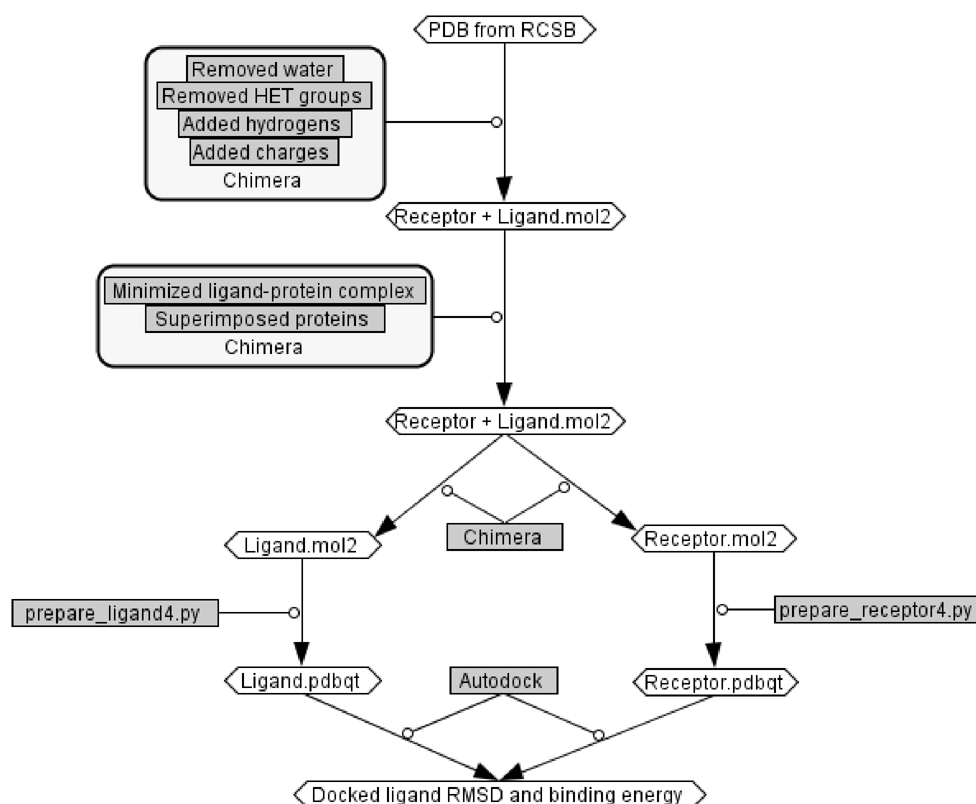

Figure 2.1: Receptor and ligand preparation protocol for redocking ligands from crystal structuresinto their original proteins.

The ligands of the 52 CAII-inhibitor complex structures were prepared for docking with AutoDockTools4 with the prepare_ligand4.py script. For the library of modified estradiol ligands, 2D structures were drawn with Chemsketch and the SMILES generated from this program was used to generated 3D .mol2 structures. The Online SMILES Translator service provided by the CADD Group and the National Cancer Institute (NCI) was used to generate 3D .mol2 structures (170).Hydrogens were added to all ligands with VEGA with the charge of the the zinc-binding nitrogen set to -1. The ligands were subjected to a conformational search of 1000 steps in VEGA(171). The systematic method with the SP4 forcefield and AMMP-MOM charges were used for the conformational search and minimization (20 steps, Toler = 0.01) and saved as .pdb files. The compounds were then saved in .mol2 format with Chimera. Finally, the ligands were prepared for docking with AutoDockTools4 with the prepare_ligand4.py script. The charge of the zinc-binding nitrogen was changed to 0.800 (0.800 NA) and the charge of the

hydrogen of the zinc-binding nitrogen was changed to -0.300 (-0.300 HD). This change improved docking conformations by constraining docking simulations to correctly form bonds between the sulfonamido nitrogen of the ligand and the zinc ion, as well as the hydrogen bond between the sulfonamido nitrogen's hydrogen and Thr199 in the docked conformations.

Docking

Speed and accuracy are two concerns as the potential size of the sampling space of a flexible ligand and a flexible protein upon binding to be measured by a scoring function grows exponentially with the number of rotatable bonds of the ligand and protein (161). One way of attempting to model protein flexibility while maintain reasonable accuracy and speed is by docking flexible ligands into the rigid structures of multiple protein receptors of the same protein. This method, also known as ensemble docking, has the advantages of being easily interpreted, having a moderate computational cost and incorporating side-chain and backbone flexibility associated with the set of proteins (196). The method however does not quantify conformational changes of the receptor or assess the effect of the ligand on the protein (196). An ensemble docking protocol was followed whereby multiple protein structures of tubulin, CAII and CAIX were used as protein receptors(196).

For the superimposed tubulin receptors, a bounding box was centered at 119.21, 89.441,6.074 for the X, Y and Z co-ordinates respectively. The size of the box was 50x50x50 with a grid spacing of 0.375 Å. For the superimposed CA receptors, a bounding box was centered at -1.852, 6.047,13.611for the X, Y and Z co-ordinates respectively. For Autodock, the Lamarckian genetic algorithm for conformational searching was used in Autodock with the ga_pop_size and ga_num_evals parameters set to 250 and 2500000 respectively. 30 runs were carried out for each ligand. For Autodock Vina, docking studies were carried out with a bounding box that encompassed the entire protein. Exhaustiveness was set to 35 with the rest of the parameters set on default(172).

General *in vitro* materials

Cell lines

The effect of selected novel compounds was investigated on tumorigenic MCF-7 breast cancer cells, tumorigenic, metastatic MDA-MB-231 breast cancer cells and non-tumorigenic MCF-12A breast cells. The MCF-7 and MDA-MB-231 cell lines are derived from a pleural effusion of human breast adenocarcinoma. The estrogen receptor positive MCF-7 cell line was provided by Highveld Biological (Pty) Ltd, Sandringham, Johannesburg, South Africa. The estrogen receptor negative MDA-MB-231 cell line was provided by Microsep (Pty) Ltd Johannesburg, RSA. MCF-7cells are hormone-dependent tumorigenic cells and MDA-MB-231 cells lack the expression of estrogen- and progesterone receptors and are metastatic and invasive (197). The MCF-12A cell line is an estrogen negative non-tumorigenic epithelial cell line established from tissue taken at reduction mammoplasty from a nulliparous patient with fibrocystic breast disease that contained focal areas of intraductal hyperplasia and was obtained as a gift from Prof MI Parker (Department of Cancer Biology, University of Cape Town, Cape Town, South Africa). The SNO non-keratinizing squamous epithelium cell line and the HeLa (human epithelial cervix carcinoma) cell line were used for preliminary cell growth studies in order to determine the effects of the compounds on cell growth on non-breast epithelial cells. The SNO and HeLa cell lines were provided by Highveld Biological (Pty) Ltd, Sandringham, Johannesburg, South Africa and the HeLa cell line was purchased through Sterilab Services (Pty) Ltd, Johannesburg, South Africa, from the American Tissue Culture Collection (ATCC), Maryland, USA.

General reagents

Dulbecco's minimum essential medium eagle (D-MEM), Trypsin-EDTA and crystal violet were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were obtained through Sterilab Services (Kempton Park, Johannesburg, South Africa). Penicillin, streptomycin and fungizone were purchased from Highveld Biological. All other chemicals (unless specified otherwise) were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Synthesis of novel compounds

The synthesis of the lead compounds was outsourced to iThemba Pharmaceuticals (Pty) Ltd (Modderfontein, Gauteng, South Africa). The company is an emerging drug discovery company that is founded by researchers and eminent academics from around the world with investments from Biotechnology Regional Innovation Centres (BRICs), LIFElab and BioPAD.

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Organic solvents of A.R. grade were used as supplied. Anhydrous N,N-dimethylformamide and N,N-dimethylacetamide were purchased from Aldrich and stored under a positive pressure of N_2 after use. Tetrahydrofuran was distilled from sodium. Sulfamoyl chloride was prepared by an adaptation of the method of Appel and Berger and stored in a tightly sealed container in the fridge (35). Chromatography was performed on silica gel (70–230 mesh; Macherey Nagel, Bethlehem, PA, USA). Thin layer chromatography was performed on Alugram® SIL G/UV254 aluminum backed plates from Macherey-Nagel GmbH & Co. (Neumann-Neander-Straße, Düren, Germany). Products were visualized with basic potassium permanganate solution. 1H NMR spectra were recorded in deuterated chloroform solution (unless otherwise indicated) with a Varian 400 NMR spectrometer at 400 MHz. Chemical shifts are reported in parts per million (ppm, d) relative to tetramethylsilane as an internal standard.

General *in vitro* methods

Cell culture procedures

Cell were grown and maintained in 25cm^2 tissue culture flasks in a humidified atmosphere at 37°C, 5 % CO_2 in a Forma Scientific water-jacketed incubator (Ohio, United States of America). MCF-7 cells were cultured in DMEM and supplemented with 10% heat-inactivated bovine fetal calf serum (FCS) (56 °C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l). MCF-12A maintenance medium consisted of a 1:1 mixture of DMEM and Ham's-

F12 medium, 20ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 µg/ml insulin and 500 ng/ml hydrocortisone, supplemented with 10% heat-inactivated FCS (56 °C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

Phosphate buffered saline (PBS) was prepared by diluting a ten times concentrated solution consisting of 80 g/l NaCl, 2 g/l KCl, 2 g/l KH_2PO_4 and 11.5 g/l Na_2HPO_4 (purchased from Merck (Munich, Germany)) to a 1 times concentrated solution. The diluted PBS solution was autoclaved (20 min, 120 °C, 15 psi) before use.

Hydroxymethylaminomethane (Tris) buffered saline (TBS) was prepared by diluting a ten times concentrated solution (pH = 7.4) consisting of 61 g/l Tris (purchased from Sigma Chemical Co. (St. Louis, MO, USA)) and 90g/l NaCl, (purchased from Merck (Munich, Germany) to a 1 times concentrated solution. The pH was adjusted to 7.4 with 6 N HCl.

Growth medium of the cells were replaced at one to three day intervals. When confluent, cells were trypsinized by removing the growth medium, washing with sterile PBS and incubating in trypsin/versene for ±10 min or when the cells appeared round and detached easily. Trypsin solution was removed and the tissue culture flask was gently tapped against the hand in order to detach the cells. The detached cells were resuspended in fresh medium and either divided into subcultures, used in experiments or frozen away in cryotubes in a -70 °C freezer. The freeze medium consists of 10% growth media, 10% dimethyl sulfoxide (DMSO) and 80% FCS.

Aseptic techniques were applied throughout, with all work being carried out in a laminar flow cabinet from Labotec (Midrand, South Africa), all solutions were filtered-sterilized (0.22 µm pore size) and all glassware and non-sterile equipment sterilized by autoclaving (20 min, 120 °C, 15 psi).

In vitro experimental procedures

For experiments, cells were seeded in 96-well (5000 cells per well) tissue culture plates, on heat-sterilized coverslips in 6-well culture plates (350 000 cells per well) in 25 cm^2 tissue culture flasks (750 000 cells per flask) or in 75 cm^2 tissue culture flasks (2 000 000 cells per flask). Cells were incubated for 24 h to allow for attachment after which medium was removed and the cells were exposed to the newly synthesized compounds dissolved in DMSO with a final concentration of DMSO not greater than 0.01% *v/v*.Cells were harvested by tripsinization as described above and were counted by making use of a haemocytometer as desribed by Freshney (1994) (198). 20 µL of the suspended cells where mixed with 80 µL PBS and 100 µL Trypan blue to give a concentration of cells with 10 times dilution factor. Dead cells take up the dye and are consequently stained blue, which is then left uncounted.
The number of viable cells per ml was determined by:
Cells/ml = Average count of viable cells in the corner squares x dilution factor x 10^4

Analytical experimental protocols

Spectrometry: Cell growth

Quantification of fixated monolayer cells was spectrophotometrically determined employing crystal violet as a DNA stain. Staining cell nuclei of fixed cells with crystal violet allows for rapid, accurate and reproducible quantification of cell number in cultures grown in 96-well plates (199, 200). Absorbance of the dye measured spectrophotometrically at 570nm corresponds to cell numbers. According to Berry *et al.* (1996) crystal violet staining of samples containing an abnormally high proportion (>30%) of stationary binucleated cells yield higher cell concentrations than trypan blue or Coulter counter methods(201). MCF-7 (202)and MCF-12A(203) cells contain less than 1% stationery binucleated cells and will therefore not lead to anomalous results. The MDA-MB-231 cell line is aneuploid (modal number = 64, range = 52 to 68), with chromosome counts in the near-triploid range(204). The SNO non-keratinizing

squamous epithelium cell line is a continuously growing and stable tumor cell line (205, 206). The HeLa cell line is aneuploid (Modal number = 82; range = 70 to 164)(207).

Dose-dependent studies were conducted in order to determine the growth inhibitory effect on the various cell lines of the newly synthesized compounds. Time-dependent studies were carried out at intervals of 24 h, 48 h and 72 h in order to observe the progression of cell growth over time in response to exposure of the compounds.

Materials

Glutaraldehyde, crystal violet and Triton X-100 were purchased from Merck (Munich, Germany).

Methods

Exponentially growing MCF-7, MDA-MB-231 and MCF-12A cells were seeded in 96-well tissue culture plates at a cell density of 5000 cells per well. Cells were incubated at 37 °C for 24 h to allow for attachment. After 24 h attachment the medium was discarded and the cells were exposed to the desired concentrations of the newly synthesized compounds as well as the vehicle control (DMSO at 0.01% v/v). The exposed and control cells were incubated for 24 h, 48 h or 72 h before the assay was performed. A baseline measurement was obtained before exposure in order to determine the starting amount of cells.

After 24 h, 48 h or 72 h exposure, the medium was discarded and 100 µL of 1% glutaraldehyde (in PBS) was added to each well and incubated at room temperature for 15 min. The glutaraldehyde was discarded and 100 µL 0.1% crystal violet (in PBS) was added and left at room temperature for 30 min. The crystal violet was discarded and the microtiter plates immersed under running tap water for 10 min and left overnight to dry. The stained cells were solubilized by adding 200 µL 0.2% Triton X-100 and incubated at room temperature for 30 min. 100 µL of the solution was transferred to a clean micrometer plate and the absorbance was read

at 570 nm with an EL_x800 Universal Microplate Reader from Bio-Tek Instruments Inc. (Vermont, United States of America).

Spectrophotometry: Cell viability

Quantification of plasma membranedamage gives an indication of cell viability (208). Lactate dehydragenase (LDH) is present in all cell types and is rapidly released into the cell culture medium upon damage of the plasma membrane(208). A lactate dehydrogenase colorimetric assay was utilized to measure the ability of extracellular LDH to oxidize lactate to reduce nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH). The LDH cytotoxicity assay kit utilizes 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) to react with NADH produced by lactate from LDH. The intensityof the generated color correlates directly to the amount of released LDH as a result of a damaged plasma membrane. Dose-dependent studies were carried out in order to determine the effect of the compounds on cell viability on the various cell lines. Time-dependent studies were conducted at intervals of 24 h and 48 h.

Time-dependent studies were performed on the GI_{50} concentrations of the newly synthesized compounds at intervals of 24 h and 48 h in order to observe the progression LDH formation over time in response to exposure of the compounds.

Materials

A lactate dehydrogenase Cytotoxicity Assay Kit from BioVision Inc. (Mountain View, California, USA), was purchased through BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa).

Methods

Exponentially growing MCF-7, MDA-MB-231 and MCF-12A cells were seeded in 96-well tissue culture plates at a cell density of 5000 cells per well. Cells wereincubated at 37 °C for 24 h to allow for attachment. After 24 h attachment the medium was discarded and the cells were exposed to the desired concentrations of the newly synthesized compounds, as well as the vehicle control (DMSO at 0.01% *v/v*). Background-controls were also included by adding only growth medium to the plates in order to subtract the effects that the medium has on the assay itself. The vehicle-treated controls, background-controls and compound-treated cells were incubated for 24 h and 48 h before the assay was performed.

A positive control for LDH production by each cell line was included by adding 10 µL of lysis buffer 30 min before the end of the 24 h and 48 h exposure times. After the 24 h and 48 h exposure, 10 µL of each sample was transferred to a new plate and incubated for 30 min at room temperature with the WST-8 reagent. The absorbance was read at 450 nm with 630 nm as reference with an EL_x800 Universal Microplate Reader from Bio-Tek Instruments Inc. (Vermont, United States of America). The cytotoxicity was calculated as follows:

$$Cytotoxicity\ (\%) = \frac{TS - BC}{PC - BC} * 100$$

TS = Test Sample

BC = Background Control

PC = Positive Control

Light and Fluorescent microscopy: Polarization-optical differential interference contrast and Hoechst 33342, acridine orange and propidium iodide fluorescent staining

Polarization-optical differential interference contrast (PlasDIC) is a polarization-optical transmitted light differential interference contrast method from Zeiss where linearly polarized light is only generated after the objective(209). PlasDIC light microscopy was usedto recognize and display the thickness of cells in relation to other cells in order to gain insight on the effects of the newly synthesized compounds on morphological changes. Fluorescent microscopy was

employed to differentiate between viable, apoptotic, autophagic and oncotic cells. A dual fluorescent dye staining method was developed utilizing acridine orange (green) and Hoechst 33345 (blue) fluorescent dyes. Acridine orange is a lysosomotropic fluorescent compound that serves as a tracer for acidic vesicular organelles including autophagic vacuoles and lysosomes (210). Cells undergoing autophagy will have an increased tendency for acridine orange staining when compared to viable cells, however, acridine orange is not a specific marker for autophagy and therefore other techniques are needed to verify the appearance of increased autophagic activity. Hoechst 33342 is a fluorescent dye that can penetrate intact cell membranes of viable cells and cells undergoing apoptosis and stain the nucleus. Time-dependent studies were carried out at intervals of 24 h and 48 h.

Materials

bisBenzimide (Hoechst 33342), acridine orange and propidium iodide were purchased from Sigma-Aldrich (St. Louis, United States of America).

Methods

Exponentially growing MCF-7, MDA-MB-231 and MCF-12A cells were seeded at 350 000 cells per well in 6-well plates. After 24 h attachment the medium was discarded and the cells exposed to the the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h or 48 h. After the exposure time, 0.5 mL of Hoechst 33342 solution (3.5 µg/mL in PBS) was added to the medium to give a final concentration of 0.9 µM and incubated for 30 min at 37 °C in a CO_2 incubator.After 25 min, 0.5 mL of acridine orange solution(4 µg/mL) were added to the medium to give a final concentration of 1 µg/mL and incubated for 5 min at 37°C.After 30 min, the medium was removed and the cells were carefully rinsed three times with PBS before being immersed in clean PBS (1 mL). The cells were examined with a Zeiss inverted Axiovert CFL40 microscope and Zeiss Axiovert MRm monochrome camera under Zeiss Filter 2 for Hoechst 33342 (blue) stained cellsand Zeiss Filter 9 for acridine orange-stained cells (green). In

order to prevent fluorescent dye quenching, all procedures were performed in a dark room (Carl Zeiss (Pty) Ltd., Johannesburg, South Africa).

Confocal microscopy: Immunofluorescent morphological observation of tubulin architecture

Confocal microscopy was employed to observe the effects of the new compounds on the cytoskeletal microtubule architecture of control and treated MCF-12A, MCF-7 and MDA-MB-231 cells. Morphological observations of tubulin architecture were performed after 24 h of exposure to the newly synthesized compounds.

Materials

A primary monoclonal mouse anti-tubulin alpha antibody from IMGENE (Alexandria, VA, USA) (cat no. IMG-80196) was purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa) and the Alexa Fluor-488, anti-mouse IgG H+L secondary antibody from Invitrogen (Carlsbad, CA, USA) (cat no. A21202) was purchased from The Scientific Group (Johannesburg, South Africa).

Methods

Exponentially growing MCF-7, MDA-MB-231 and MCF-12A cells were seeded at 350 000 cells per well in 6-well plates. After 24 h attachment the medium was discarded and the cells exposed to the the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h. Cells were washed with a cytoskeletal bubber (CB) (60 mM 1,4 piperazinediethanesulfonic acid, 27 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM ethylene glycol tetraacetic acid, 4 mM magnesium sulphate heptahydrate, pH = 7.0) and fixated with 0.3% glutaraldehayde at 37˚C for 15 min. In order to stain the intracellular components, cellular

membranes were permeabilized with one percent Triton X-100 in CB buffer. Unreacted aldehydes interfere with the binding of the primary antibody to alpha-tubulin, thus, unreacted aldehydes were removed by treating the cells with sodium borohydride in water (1 mg/mL) for 10 min. Non-specific binding was inhibited by immersing the cells in 2% bovine serum albumin (BSA). Alpha tubulin was marked with anti-alpha tubulin antibodies (raised in mice) by incubating the cells with the antibodies for 90 min together with one percent Triton X-100 and 2% BSA. Cells were washed after primary antibody staining and counter stained with an anti-mouse Ig1 Alexa-Fluor 488 secondary antibody for 90 min. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI). Stained cells were viewed with a Zeiss 510 META confocal laser microscope(Carl Zeiss (Pty) Ltd., Johannesburg, South Africa).

Transmission electron microscopy: Morphological examination of intracellular ultrastructure

Transmission electron microscopy (TEM) was used to determine the ultra structure of intracellular components of exposed and control MCF-12A, MCF-7 and MDA-MB-231cells. TEM is an imaging technique whereby a beam of electrons is focused onto a specimen. The electron beam is partially transmitted through the very thin specimen and carries information about the inner structure of the specimen (211). Morphological observations via TEM was performed after 24 h and 48 h of exposure to the newly synthesized compounds.

Materials

Aqueous osmium tetroxide, glutaraldehyde, phosphate buffer quetol, Reynolds' lead citrate, aqueous uranyl acetate were purchased by the Electron Microscopy Unit of the University of Pretoria from Merck Co. (Munich, Germany). The Multi-purpose Philips 301 transmission electron microscope of the Electron Microscopy Unit of the University of Pretoria was used for viewing the prepared samples.

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 750 000 cells per $25cm^2$ flask. After 24 h attachment the medium was discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h or 48 h. After 48h cells were trypsinized and resuspended in 1 mL growth medium in 1.5 mL eppendorfs. Cells were fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4-7.6) for 1 h and rinsed 3 times for 5 min each with 0.075 M phosphate buffer. Thereafter the cells were fixed in 0.25% aqueous osmium tetroxide for 30 min and rinsed three times in distilled water in a fume cupboard. The samples were dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90%, 100%, 100%, 100%) and infiltrated with 30% quetol in ethanol for 1 h. The samples were then filtrated with 60% quetol for 30 min and thereafter pure quetol for 4 h. The samples were polymerized at 60 °C for 36 h or longer. Ultra-thin sections were prepared with a microtome and mounted on a copper grid. The samples were contrasted with 4% uranyl acetate for 10 min and rinsed with water. Enhancement of contrast was obtained by placing samples in Reynolds' lead citrate for 2 min and rinsing with water. Samples were viewed with a Multi-purpose Philips 301 TEM (South African Philips (Pty) Limited, Martindale, Johannesburg, South Afica) from the Microscopy Unit of the University of Pretoria.

Flow cytometry: Cell cycle progression

Flow cytometry was employed to measure the DNA content of exposed and control cells in order to monitor the effect on cell cycle progression in MCF-12A, MCF-7 and MDA-MB-231cells. Analysis was conducted by ethanol fixation and propidium iodide staining of cells. Propidium iodide was used to stain the nucleus in order to determine the amount of DNA present. The amount of DNA present correlates with the stages of the cell cycle during cell division. Time-dependent studies were conducted at intervals of 6 h, 12 h, 18 h, 24 h and 48 h. Information from the time-dependent study yielded insight into the progression of cell cycle events over time in vehicle-control and treated cells.

Materials

99.9% ethanol was purchased from Merck Co. (Munich, Germany). Propidium Iodide was purchased from Sigma-Aldrich (St. Louis, United States of America). Bovine Pancreas Ribonuclease A (RNaseA) from US Biologicals (Swampscott, Massachusetts, USA) was purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 350 000 cells per well in 6-well plates. After 24 h attachment the medium was discarded and the cells were exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for the desired period of time. After the allotted time of treatment, cells were trypsinized and 500 000 cells were resuspended in 1 mL growth medium. One million cells were centrifuged for 5 min at 300xg. The supernatant was discarded and the cells were resuspended in 200 µL of ice-cold PBS containing 0.1% FCS. Ice-cold 70% ethanol (4 mL) was added in a drop wise manner and the cells were stored at 4ºC for 24 h. After 24 h, the cells were pelleted by centrifuging them at 300xg for 5 min. The supernatant was removed and the cells were resuspended in 1 mL of PBS containing propidium iodide (40 µg/mL). Sample were incubated at 37ºC for 45 min.

Propidium iodide fluorescence (relative DNA content per cell) was measured with a fluorescence activated cell sorting (FACS) FC500 System flow cytometer (Beckman Coulter South Africa (Pty) Ltd) equipped with an air-cooled argon laser excited at 488nm. Experiments were conducted in triplicate and data from at least 10 000 cells was analyzed with Cyflogic (CyFlo Ltd, Turku, Finland) and with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia).

Flow cytometry: Apoptosis detection

Flow cytometry was employed to analyze apoptosis in MCF-12A, MCF-7 and MDA-MB-231 cells. Cells were stained with FITC-conjugated Annexin V to measure the translocation of the membranephosphatidylserine (PS). PS translocation is associated with apoptotic processes. Time-dependent studies were carried out at intervals of 6 h, 12 h, 18 h, 24 h and 48 h.

Materials

Annexin V-FITC Kit was purchased from BIOCOM biotech Pty (Ltd) (Clubview, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 350 000 cells per well in 6-well plates. After 24 h attachment the medium was discarded and the cells were exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for the desired period of time. After the allotted time of treatment, cells were trypsinized and 500 000 cells were resuspended in 1 mL of 1x binding buffer (pH7.4), HEPES 100.0 mM, NaCl 1.5 M, KCl 50.0 mM, $MgCl_2$ 10.0 mM, $CaCl_2$ 18.0 mM) and centrifuged at 300×g for 10 min. The supernatant was removed and the cells were resupended in 100 μL of 1x Binding buffer. 10 μL of Annexin V-FITC was added and the samples were incubated for 15 min in the dark at room temperature. After 15 min the cells were washed by adding 1 mL of 1x binding buffer and centrifuged at 300×g for 10 min. The supernatant was carefully removed and the cells were resuspended in 500 μL of 1x binding buffer solution.

Aannexin V fluorescence (apoptotic cells) were measured with a FACS FC500 System flow cytometer (Beckman Coulter South Africa (Pty) Ltd) equipped with an air-cooled argon laser excited at 488 nm. Experiments were conducted in triplicate and data from at least 10 000 cells were analyzed with Cyflogic (CyFlo Ltd, Turku, Finland) and with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia).

Flow cytometry: Mitochondrial membrane potential detection

Mitochondrial membrane potential was monitored usingMitocapture™. Mitocapture™ is a cationic dye that accumulates andaggregates in the mitochondria of healthy cells, providing a bright red fluorescence(212). In apoptotic cells, MitoCapture™ cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential, and thus remains inthe cytoplasm in its monomer form, generating a green fluorescence (212). Time-dependent studies wereconducted at intervals of 6 h, 12 h, 18 h, 24 h and 48 h.

In order to gain further mechanistic insight into the effects that stress activated protein kinases have on mitochondrial membrane potential, cells were exposed to inhibitors the selective JNK kinase inhibitor, SP600125 (Sigma cat no: S5567) and the selective p38 MAP kinase inhibitor,SB239063 (Sigma cat no: S0569).

Materials

A Mitocapture apoptosis detection kit from BioVision Inc. (Mountain View, California, USA) was purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 350 000 cells per well in 6-well plates. After 24 h attachment the medium was discarded and the cells were exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for the desired period of time. After the allotted time of treatment, cells were trypsinized and 500 000 cells were incubated with 0.5 mL of the MitoCapture™ reagent for 15 min. Fluorescence of the dye was measured with a FACS FC500 measured with a fluorescence activated cell sorting (FACS) FC500System flow cytometer (Beckman Coulter SA (Pty) Ltd.).Data from at least 10

000 cells were analyzed with Cyflogic (CyFlo Ltd, Turku, Finland) and with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia).

Flow cytometry: Autophagy detection

Flow cytometry was employed to analyze autophagy induction in MCF-12A, MCF-7 and MDA-MB-231 cells. Cells were stained with FITC-conjugated microtubule-associated proteins 1A/1B light chain 3B membrane protein (LC3) antibodyto quantify the autophagy-related protein. LC3 is required for the formation process of autophagosomes and is the only molecule controlled by nutrient supply and deprivation in the autophagy-related gene products(145).

Materials

An FITC conjugated anti-autophagy-related (LC3) antibody from Novus Biologicals Ltd. (Cambridge, United Kingdom) was purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa). Bovine Serum Albumin (BSA) and Triton X-100 was purchased from Sigma-Aldrich (St. Louis, United States of America).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 750 000 cells per 25cm^2 flask. After 24 h attachment the medium were discarded and the cells were exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for the desired period of time. After the allotted time of treatment, cells were trypsinized and washed with ice-cold PBS. 1 000 000 cells were resuspended cells was fixed with 0.01% paraformaldehyde in PBS for 10 min and pelleted and resuspended in ice-cold PBS. In order to permeabilize the cells, ice-cold methanol (stored at -20 °C) was added in a drop wise manner. Cells were thencentrifuged and washed twice with cold PBS. Thereafter, the cells were pelleted and 500 µL of the primary antibody mixture solution (0.5 µg/mL of the anti-LC3 FITC-conjugated antibody) was added and

incubated for 2 hours at 4 °C in the dark. After 2 hours of incubation, cells were washedtwice with washing buffer (PBS/0.05% Triton/1%BSA) and were measured with a FACS FC500 System flow cytometer (Beckman Coulter SA (Pty) Ltd.).Data from at least 10 000 cells was analyzed with Cyflogic (CyFlo Ltd, Turku, Finland) and with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia).

Flow cytometry: Hydrogen peroxide and superoxide

Hydrogen peroxide generation was assessed using 2,7-dichlorofluorescein diacetate (DCFDA), a non-fluorescent probe, which, upon oxidation by H_2O_2, is converted to the highly fluorescent derivative 2,7-dichlorofluorescein (DCF)(213).Superoxide generation was assessed using hydroethidine (HE). HE is oxidized by superoxide and not by hydroxyl radicals, singlet O_2, H_2O_2 or nitrogen radicals to a red fluorescing compound (214)

Flow cytometry was utilized to gain quantitative information of intracellular DCF formation and oxidized intracellular HE formation. Data from at least 10 000 cells were analyzed with CXP software (Beckman Coulter South Africa (Pty) Ltd).Time-dependent studies were conducted at intervals of 6 h, 12 h, 18 h, and 24 h in order to analyze the progression of hydrogen peroxide and superoxide formation.

Materials

2,7-dichlorofluorescein diacetate and Hydroethidine were purchased from Sigma-Aldrich (St. Louis, United States of America).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 350 000 cells per well in 6-well plates. After 24 h attachment the medium was discarded and the cells were

exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for the desired period of time. After the allotted time of treatment, cells were trypsinized and 500 000 cells were resuspended in cold PBS. The cells were incubated with 1 µM DCFDA for 25 or 10 µM HE for 15 min at 37˚C min at 37˚C. DCF fluorescence was measured using the FL1 filter and HE fluorescence was measured with the FL3 filter of the FACS FC500System flow cytometer (Beckman Coulter SA (Pty) Ltd.).Data from at least 10 000 cells was analyzed with Cyflogic (CyFlo Ltd, Turku, Finland) and with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia).

Gene expression analysis: Complimentary RNA microarray

Agilent's Human 1A Oligo 60-mer Microarray (V2) 44k slides with more than 41 000 60-mer oligonucleotide human genes and transcripts were employed to study expression changes on the MCF-12A, MCF-7 and MDA-MB-231 cell lines induced after24 h treatment of selected compounds. A dye-swop methodology with three biological replicates was employed in order to remove the effects of dye-bias on statistical analyses and make the genomic information statistically relevant.

Materials

Qiagen's Plant Mini Kit, PCR Clean-up kit and RNase-free DNase were purchased from Southern Cross Biotechnology (Pty) Ltd. (Cape Town, South Africa).Agilent's 22k and 44k 60-mer human oligo slides, Low RNA Input Fluorescent Linear Amplification Kit, 2x GEx Hybridization Buffer HI-RPM, Gene Expression (GE) Wash Buffer 1 and 2, Stabilization and Drying Solution were obtained from Agilent Technologies (Pty) Ltd. (Palo Alto, California, United States of America).Cy-3 and Cy-5 fluorescent dyes were supplied by Amersham Biosciences (Pittsburgh, United States of America).

Methods

I) RNA extraction

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells was seeded at 750 000 cells per 25cm^2 flask. After 24 h attachment the medium was discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h or 48 h. After exposure, the cells were washed twice with PBS and lysed by adding 1 mL RLT buffer (RNeasy Mini Kit) to each flask and the flask was decanted until all the cells were lysed. The lysate was pipetted into Qiashredder columns and centrifuged for 2 min at 9000xg. The flow-through was collected and 1 volume of 70% ethanol was added and gently mixed. This solution was divided into Qiagen Plant Mini Kit columns (700 µL per column) and centrifuged for 15 s at 9000xg. The flow-through was discarded. 350 µL RW1 buffer was added to each column in order to wash the column. The column was centrifuged for 15 s at 9000xg. A DNase mixture was prepared utilizing Qiagen's RNase-free DNase Set, by adding 70 µL RDD buffer to every 10 µL DNase1. 80 µL of this mixture was added in the middle of each column and left at room temperature for 15 min. Another 350 µL RW1 buffer was added and the column was centrifuged for 15 s at 9000xg. Flow-through was discarded and columns transferred to new eppendorf tubes. A series of washing steps followed. 500 µL RPE buffer was added to each tube and centrifuged for 15 s at 12000xg, discarding the flow-through afterwards and replacing the eppendorf tubes. This step was repeated. The column was the then centrifuged at 9000xg for 1 min. To elute the total RNA from the column 50 µL RNase-free water was added to the column and centrifuged for 1 min at 9000xg. The total RNA was suspended in 50 µL RNase-free water and was ready to be quantified with the Nanodrop and tested for integrity by means of electrophoresis.

II) RNA integrity

Agarose powder (0.6 g) was dissolved in 40ml RNase free water (1.5% gel). 6ml 10x 3-(N-morpholino)propanesulfonic acid (MOPS), 3.4 ml formaldehyde and 14 ml RNase free

water was added. A sample mix to be loaded in the well was prepared by adding 3µg total RNA (±8µL), 2µL tracking dye (50% glycerol, 100mM Na_2EDTA, pH 8.0, 1% SDS, 0.1% bromophenol blue 0.1%) and 1µL ethidium bromide (0.5 µg/mL final concentration). The mix was heated for 15min at 55ºC and rapidly chilled on ice afterwards. 15µL of each sample was pipetted into a well of the agarose-formaldehyde RNA gel and electrophoresis was conducted at 80mV for 45min.

III) Labelled cRNA synthesis

Agilent's Low RNA Input Fluorescent Linear Amplification Kit was used to generate fluorescently labeled cRNA. Labeled cRNA was produced using the T7 polymerase and purification of labeled cRNA was conducted using Qiagen's RNeasy Mini kits. For each sample, 2000 ng of total or polyA RNA was added to a 1.5 mL microcentrifuge tube and filled with RNase free water to a final volume of 8.3 µL or less.

Spike A Mix (2.0 µL)containing the positive control mRNA for the cyanine 3-CTP dye reactions and the Spike B Mix (2.0 µL)containing the positive control mRNA for the cyanine 5-CTP dye reactions were added to their respective samples containing the extracted total RNA from either the vehicle-treated controls or the compound-treated experiments.1.2 µL of T7 Promoter Primer (from the Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color) was added to each sample and the primer and the template were denatured by incubating the reaction at 65 °C in a circulating water bath for 10 minutes. After 10 min the reactions were placed on ice and incubated for 5 minutes.

For each sample 4 µL 5X First Strand Buffer, 2 µL 0.1 M Dithiothreitol (DTT), 1 µL 10 mM dNTP mix (10mM dATP, dCTP, dGTP, dTTP), 1 µLMoloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and 0.5 µLRNase inhibitor (RNaseOUT)was added and incubated at 40 °C in a circulating water bath for 2 hours. After 3 hours the samples were incubated at 65 °C in a circulating water bath for 15 minutes after which it was incubated on ice for 5 minutes.

Samples were briefly centrifuged to drive down any precipitation. For each sample 15.3 µLnuclease-free water, 20 µLof 4X Transcription Buffer,6µL of 0.1 M DTT, 8 µL of NTP mix, 0.5 µL 50% Polyethylene glycol (PEG) 6.4 RNaseOUT, 0.6 µL Inorganic pyrophosphatase, 0.8 µL T7 RNA Polymerase and 2.4µLof eitherCyanine 3-CTP or cyanine 5-CTP. The samples were incubated at 40 °C in a circulating water bath for 2 hours.

The labeled cRNA was purified using Qiagen's RNeasy mini spin columns. To each sample, 20 µL of nuclease-free, 350 µL of Buffer RLT and 250 µL of ethanol (100% purity) were added and mixed thoroughly by pipetting. This solution was pipette onto the RNeasy mini spin columns columns (700 µL per column) and centrifuged for 15 s at 9000xg. The flow-through was discarded and 500 µL RPE buffer was added to each tube and centrifuged for 15 s at 12000xg, discarding the flow-through afterwards and replacing the eppendorf tubes. This step was repeated. The column was the then centrifuged at 9000xg for 1 min without anything added to column in order to remove all residual liquid. In order to elute the total RNA from the column 35 µL RNase-free water was added to the column and centrifuged for 1min at 9000xg. The labeled cRNA was suspended in 35 µL RNase-free water and was quantified with the Nanodrop.

V) Hybridization of Cy-dye labeled cRNA

Cleaned cRNA was hybridized to Agilent Human 1A (V2) oligonucleotide 44K microarray slides according to the manufactures guidelines using Agilent's 2x GEx Hybridization Buffer HI-RPM in Agilent's SureHyb chambers. A hybridization mixture was prepared by adding equal amounts of Cy-3 and Cy-5 labeled cRNA (825 ng), 11 µL 10X blocking Agent, 2.2 of 25x Fragmentation Buffer to a final volume of to a final volume of 55 µL. The samples were incubated at 60 °C for exactly 30 minutes to fragment the RNA. After 30 min, 55 mL of the 2x Hybridization Buffer was added to each sample.The backing slides were placed in Agilent's microarray hybridization chambers and the above prepared solution was pipette (100 µL) onto each grid (four grids on one slide).

The assembled slide chamber was placed in Agilent's hybridization ovens set to 65 °C and 10 rpm.The samples were hybridized atfor 17 hours. Afterwards the slides were washed twice for

1 min in Falcon tubes containing Agilent's Gene Expression Wash Buffer 1 at room temperature and once in Falcon tubes containing Agilent's Gene Expression Wash Buffer 2 at 37 °C for 1 min. The slide was transferred to acetonitrile for 1 min and then subsequently to Agilent's Stabilization and Drying Solution to help prevent ozone bleaching of the Cy-fluorochromes. The slides were gently removed from the Stabilization and Drying Solution and scanned immediately.

VI) Scanning of Agilent microarray slides

Slides were scanned with the Axon Genepix 4000B Scanner (Molecular Devices, USA) provided by the ACGT Microarray Facility at the University of Pretoria.

VII) Spotfinding

Spotfinding was performed using Genepix Pro 6.1 (Molecular Devices Corporation, Sunnyvale, California USA). GenePix Pro 6.1 uses a set of proprietary feature-finding algorithms to find circular features. Every pixel in a region around the feature is examined by a local alignment algorithm and assigned to feature or background. Global alignment algorithms determine the translation, rotation and skew of blocks of features. Saturated spots, spots with an uneven background, non-uniform spots and spots with a low intensity vs back ground ratio were removed from further analysis by excluding the spots that satisfied the following parameters. The excluded spots were assigned as "Bad".

Saturated spots:

[F532 % Sat.] > 30 And

[Ratio of Means (635/532)] > 0.75 Or

[F635 % Sat.] > 30 And

[Ratio of Means (635/532)] < 1.3333

Spots with an uneven background:

([B635 Mean] > (1.5*[B635 Median]) Or
[B532 Mean] > (1.5*[B532 Median])) And
([B635 Median] > 40 Or
[B532 Median] > 40)

Non-uniform spots:
[Ratio of Medians (635/532)] > (4.0*[Rgn Ratio (635/532)]) Or
[Ratio of Medians (635/532)] < (0.25*[Rgn Ratio (635/532)])

Low intensity vs back ground ratio:
[% > B635+2SD] < 10 Or [% > B532+2SD] < 10

VIII) Limma statistical analysis

Statistical analysis after spotfinding was conducted using Limma with the LimmaGUI interface (215, 216). Background correction was done with the normal+exponential (Normexp) convolution model to observed intensities. The normal part represents the background and the exponential represents the signal intensities (216). The Normexp offset value was set to 25. A value of 25 maximized the df.prior. df.prior is the numeric vector giving empirical Bayes estimated degrees of freedom associated with s2.prior for each gene. s2.post is the numeric vector giving posterior residual variances. A maximized df.prior is optimal and will allow for greater power to detect differentially expressed genes (216). Spot quality weighting was performed and Genepix Flag weightings that were flagged as "Bad" were excluded from further analysis. Normalization within arrays was performed to remove dye-bias at higher and lower intensities by normalizing M-values (log-ratios) with the Global Loess method (217). The M-value (M) represents a log2-fold change between two or more experimental conditions. The A-value (A) is the average log2-expression level for a gene across all the arrays and channels in the experiment.

The M-value is calculated as follows:
M = log2(Cy5)/Cy3) (Cy5/Cy3 are the normalized emission intensities of the spot)

The A-value is calculated as follows:

A = (log2(Cy5)*Cy3))/2

Aquantile normalization between arrays was performed in order to normalize expression intensities so that the intensities or log-ratios have similar distributions across a series of arrays (217). Aquantile normalization ensures that the A-values (average intensities) have the same empirical distribution across arrays leaving the M-values (log-ratios) unchanged (217). The Least squares linear model fit method was employed and the *P*-values were adjusted for multiple testing utilizing the Benjamini and Hochberg's ste*P*-up method for controlling the false discovery rate (218). Genes that had a *P*-value of less than 0.05 were considered statistically significantly differentially expressed and were included in further analyses.

IX) Gene expression analysis

Biological interpretation and functional analysis of gene lists were performed by mapping differentially expressed genes to biochemical pathways and Gene Ontology (GO) categories using Gene Annotation Co-occurrence Discovery (GENECODIS) (219). GENECODIS is a web-based tool for finding sets of biological annotations that frequently appear together and are significant in a set of genes (219). In order to determine common genes that were affected by the compounds between cell lines as well as the different compounds, differentially expressed gene lists were compared utilizing GeneVenn (220). GeneVenn is a simple, web-based application creating Venn diagrams from two or three gene lists.

Gene expression analysis: Reverse transcription quantitative polymerase chain reaction

Reverse transcription quantitative polymerase chain reaction(RTq-PCR) technology is an accurate and practical method for gene level mRNA measurement(221). It is a good tool that can be used to independently validate cRNA microarray results as well as measure mRNA expression on its own. SABiosciences™ (Maryland, USA) apoptosis and cell cycle Human RT^2 profiler Arrays were used to detect difference in mRNA expression levels between treated and vehicle-treated samples. The PCR arrays come with validated primers to 84-pathway specific

genes(222). Each plate contains 5 house-keeping genes, a genomic DNA control, positive PCR control and reverse transcription controls. First strand cDNA synthesis was conducted as prescribed by the manufacturer. Each primer set is validated for use with SABiosciences' SYBR Green Master Mixes.

Materials

Qiagen's Plant Mini Kit, PCR Clean-up kit and RNase-free DNase were purchased from Southern Cross Biotechnology (Pty) Ltd. (Cape Town, South Africa).Human RT^2 profiler Arrays (384 wells) for apoptosis (cat no: PAHS-012G) and cell cycle (PAHS-020A) analysis of mRNA levels, the RT^2 First Strand Kit for first strand cDNA synthesis and the RT^2 qPCR Master Mix kits for amplification and labeling of cDNA were purchased from SABiosciences™ (Frederick, Maryland, USA) were purchased from Whitehead Scientific (Pty) Ltd. (Johannesburg, Gauteng South Africa). The Roche LightCycler 480 at the Real-time PCR Facility at the University of Pretoria was purchased from Roche (Illovo, Johannesburg, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 750 000 cells per $25cm^2$ flask. After 24 h attachment the medium were discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 12 h or 24 h. Total RNA from vehicle-control and exposed cells of three biological replicateswere isolated and checked for purity, concentration and integrity as described above. Each 384-well plate contains four replicates of the same gene. Therefore, a plate was used for three biological repeats and one biological repeat was used as a technical replicate to proved an *n* number of four for each sample.

Genomic DNA was eliminated by preparing a Genomic DNA Elimination Mixture. 1 µg of total RNA (dissolved in 8 µL RNAse free water) was mixed with 2µL of the gDNA Elimination Buffer (5x-GE) and incubated for 5 min at 42 ºC. After incubation ha samples were immediately chilled on ice. A reverse transcriptase (RT) cocktail was prepared for four reaction by mixing 16 µL of BC3 (5x RT buffer), 4 µL P2 (Primer and external Control Mix), 8 µL RE3 (RT Enzyme Mix 3) and 12 µL RNAse free water. 10 µL of the RT cocktail was mixed the 10 10 µL Genomic DNA Elimination Mixture and incubated for exactly 15 min at 42 ºC. After 15 min the RNA was degraded by incubating the sample for 5 min at 95 ºC. 91 µL of RNAse free water was added to each reaction for a final volume of 111 µL of the First Strand cDNA Synthesis Reaction.

An Experimental Cocktail for amplification and labeling was prepared for 96 reactions. 550 µL of SABiosciences' 2x qPCR Master Mix, 102 µL of the First Strand cDNA Synthesis Reaction and 448 µL of RNAse free water were mixed together. 10 µL of the Cocktail was loaded into each of the 96 wells of the PCR plates containing the primers for specific genes. The plates were filled with all the repeats (n = 4 , 96x4=384), sealed and briefly centrifuged to drive the contents down.

The Roche LightCycler 480 was set up to have one 10 min cycle at 95 ºC in order to activate the HotStart DNA polymerase. This was followed by 45 cycles at 95 ºC for 15 seconds (for optimal reverse transcription) and 60 ºC for 1 min (for annealing) with a ramp rate of 1 ºC/second. SYBR green fluorescence was measured during the 1 min of annealing.

The acceleration of the fluorescence signal at its maximum was considered as the crossing point (CP) of the PCR reaction. The Second Derivative Maximum analysis method (set at high Confidence) available in the LightCycler 480 software package was used to identify the crossing point. The CP values were used to analyze the relative expression and SABiosciences' Excel-based PCR Array Data Analysis software.

The log fold change of a particular gene ($LogFC_{gene}$) was calculated after the CP values were normalized to the internal control (IC), hypoxanthine-guanine phosphoribosyltransferase (HGPRT).

$$LogFC_{gene} = \frac{2^{(ExperimentCP_{gene} - ExperimentCP_{IC})}}{2^{(ConrtolCP_{gene} - ControlCP_{IC})}}$$

A $LogFC_{gene}$ value of greater than one indicate a positive- or an up regulation and a $LogFC_{gene}$value of less than one indicates a a negative or down regulation. The $LogFC_{gene}$is the fold regulation value for positively regulated genes. The following formula yields the fold regulation value for negatively regulated genes:

$$\text{Fold change for negatively regulated genes} = -\frac{1}{LogFC_{gene}}$$

The P-values were calculated by a Student's t-test of the replicate for $2^{(CP_{gene}-CP_{IC})}$ for each gene in the control group and experiment groups. P- values less than 0.05 were considered statistically significantly differentially expressed.

Protein expression analysis: Protein microarray

Gene expression profiles obtained from microarray data indicate relative mRNA levels of genes associated with apoptosis, cell cycle regulation and signal transduction at the time of termination. However, gene expression does not necessarily correlate with protein expression or activity. Antibody microarrays provide a means to measure protein expression levels in whole cell extracts (223). The technique involves the spotting of a slide with a library of antibodies relevant to a particular functional pathway being studied. Proteins in whole protein extracts are labeled with fluorescent dyes and hybridized onto the antibody protein microarray. The relative fluorescence of each spot provides an indication of the relative abundance of the protein in control and treated samples. The technique will yield valuable mechanistic information with regards to signaling mechanisms being activated or abrogated in treated samples when compared to control samples (224).

Materials

Clontech's AB Microarray 500 slides (cat no: 631790), Protein Extraction and Labeling Kit (cat no: 631786 and the Ab Microarray Express Buffer Kit (cat no: 631795) were purchased from Southern Cross Biotechnology (Pty) Ltd. (Cape Town, South Africa). Pierce Biotechnology's

(Rockford, Illinois, USA)bicinchoninic acid (BCA) Protein Assay Reagent Kit and Thermo Fisher Scientific's (Waltham, Massachusetts, USA) Zeba Spin Desalting Columns, 7K MWCO were purchased through Separations (Johannesburg, Gauteng, South Africa). Cy5 mono-Reactive Dye (Cat no: PA25001) and Cy3 mono-Reactive Dye Packs (Cat no: PA23001) was purchased from GE Healthcare (Johannesburg, South Africa).

Methods

I) Protein extractionand concentration determination

Exponentially growing MCF-7 and MDA-MB-231cells were seeded at 2 000 000 cells per 75 cm^2 flask. After 24 h attachment the medium was discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h. After exposure, cells were washed 4 times with PBS and then trypsinized. The cells were collected and centrifuged. The supernatant was removed and the pellet was frozen at -80 °C and then weighed.20 µL of Extraction/Labeling Buffer was added for every 1mg of cells and incubated for 10 min. The sample was then centrifuged at 10,000 x g for 30 min at 4°C. Afterwards, the supernantant was transferred to a new 1.5 mL tube and the protein concentration was measured with BCA (Pierce's BCA Protein Assay Reagent Kit). Bovine serum albumin from the kit was used to make various standard concentrations of protein ranging from 0 mg/mL to 2.5 mg/mL. The BCA kit consists of two parts. Reagent A contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide. Reagent B is 4% cupric sulfate. A working solution was freshly made by mixing 50 parts of Reagent A and 1 part of Reagent B. 10 µL of each protein sample was pipetted into a 96-well plate and mixed with 200 µL of the working solution. The samples were covered with foil and incubated for 30 min at 37 °C. The absorbance at 570 nM of each sample was measured with an EL_x800 Universal Microplate Reader from Bio-Tek Instruments Inc. (Vermont, United States of America). The linear relationship between the absorbance and the standard concentrations was used to determine the concentration of the protein in the control and exposed cell samples. Each cell sample was diluted with Extraction/Labeling Buffer to yield a final concentration of 1.1 mg/mL.

II) Protein labeling

Two antibody array slides were used for each cell line. There are two replicates of each protein an antibody array slide. Therefore, two biological replicates and two technical replicates provided an n number of 4. For the first biological replicate, the control was labeled with Cy3 dye (green) and the experiment was labeled with Cy5 dye. For the second biological replicate, the control was labeled with Cy5 dye (green) and the experiment was labeled with Cy3 dye. This two-slide dye-swop methodology is used to remove any dye bias that may result due to differences in Cy-dye incorporation. Fresh Cy3 and Cy5 mono-Reactive Dyes were reconstituted with 110 µL Extraction/Labeling Buffer. 94 µL of the protein samples (1.1 mg/mL) was mixed with 6 µL of the reconstituted Cy-dyes and incubate at 4 °C for 90 min in the dark. The samples were mixed by inversion every 20 min. After 90 min, 4 µL Blocking Buffer was added and further incubated for 30 min at 4 °C in the dark. The samples were mixed by inversion every 10 min.

III) Removal of Unbound Cy-Dye and determination of Cy-dye incorporation

Zeba Spin Desalting Columns (7K MWCO) were used to desalt the samples and remove any unreacted Cy-dye. The storage solution was removed by centrifugation and the protein samples (±104 µL) was loaded onto the desalting columns and centrifuged at 1,500×g for 2 minutes. Bovine serum albumin from the BCA kit was used to make various standard concentrations of protein ranging from 0 mg/mL to 0.5 mg/mL and the protein concentration ($ProtConc_{mg/ml}$) was measured as described above. Cy3 absorbance ($A552$) and Cy5 absorbance ($A650$) were measured with a Nanodrop. Incorporation of Cy3 and Cy5 dyes into protein was calculated as follows. For Cy3, a molar extension coefficient ($\varepsilon552$) of 150,000 $M^{-1}.cm^{-1}$ was used as suggested. The average molecular weight of protein was assumed to be 60,000 Da (as suggested by the manual).

The Cy3 concentration ($Cy3Conc_{\mu M}$) in the sample was calculated as:

$$Cy3Conc_{\mu M} = \left(\frac{A552}{\varepsilon 552}\right) \times 10^6$$

The protein concentration ($ProtConc552_{\mu M}$) in the sample was calculated as:

$$ProtConc552_{\mu M} = \frac{ProtConc552_{mg/ml}}{60000\ Da} \times 10^6$$

For Cy5, a molar extension coefficient ($\varepsilon 650$) of 250,000 $M^{-1}.cm^{-1}$ was used as suggested. The average molecular weight of protein was assumed to be 60,000 Da (as suggested by the manual). The Cy5 concentration ($Cy5Conc_{\mu M}$) in the sample was calculated as:

$$Cy5Conc_{\mu M} = \left(\frac{A650}{\varepsilon 650}\right) \times 10^6$$

The protein concentration (ProtConc650_µM) in the sample was calculated as:

$$ProtConc650_{\mu M} = \frac{ProtConc650_{mg/ml}}{60000\ Da} \times 10^6$$

The following ratiosyielded information regarding the average number of coupled dye molecules for the respective dyes:

$$\frac{Cy3Conc_{\mu M}}{ProtConc552_{\mu M}}\ and\ \frac{Cy5Conc_{\mu M}}{ProtConc650_{\mu M}}$$

A ratio of 2-4 for each of these ratios was considered sufficient to proceed to hybridization.

V) Hybridization of Cy-dye labeled protein

Cy3-labeled vehicle-control protein (100 µg) and 100 µg of Cy5-labeled treated protein was added together for slide 1 (Slide 1 mix). 100 µg of Cy3-labeled treated protein and 100 µg of Cy5-labeled vehicle-control protein were added together for slide 2 (Slide 2 mix).

For each slide, 45 mL of was prepared by mixing 4.5 mL Background Reduce and 40.5 mL Stock Incubation Buffer. 5 mL of Incubation Buffer was added to each incubation chamber as well as wash chamber. Thereafter, 20 µg of Slide 1 mix was added to the incubation chambers

containing the 5 mL of Incubation Buffer for slide 1 and mixed for 10 min at room temperature in the dark. 20 µg of Slide 2 mix was added to the incubation chambers containing the 5 mL of Incubation Buffer and mixed for 10 min at room temperature in the dark.

The slides were prepared by decanting the storage buffer from the storage vial if the slides into a waste beaker. 30 mL of Stock Incubation Buffer was added into the storage vial and slowly inverted for 10 min. The Stock Incubation Buffer was decanted and fresh Stock Incubation Buffer (20 mL) was added to the storage vial. The storage vial was slowly inverted for 10 min. After the washing of the slides, a slide was placed into each incubation tray and incubated at room temperature for 40 min while slowly rocking the incubation trays.

After 40 min, 5 mL of Wash Buffer A was added to the wash chambers already containing Incubation Buffer. The slides were gently transferred to the wash chamber and incubated for 5 min. The buffer from each wash chamber was gently removed and replaced with 5 mL of Wash Buffer B and incubated for 5 min at room temperature. Wash buffer B was then removed and replaced with 5 mL of Wash Buffer C and incubated at room temperature for 5 min. The slides were then transferred to 50 mL tubes containing deionized water and the transferred again to an empty 50 ml tube. The slides were then dried by centrifuging the tubes for 5 min at 1000xg at room temperature.

Slides were scanned with the Axon Genepix 4000B Scanner (Molecular Devices, USA) provided by the African Centre of Gene Technology (ACGT) Microarray Facility at the University of Pretoria.

VI) Spotfinding

Spotfinding was performed using Genepix Pro 6.1 (Molecular Devices Corporation, Sunnyvale, California USA). GenePix Pro 6.1 uses a set of proprietary feature-finding algorithms to find circular features. Saturated spots, spots with an uneven background, non-uniform spots and spots

with a low intensity vs back ground ratio were removed from further analysis by assigning them as "Bad".

VII) Limma statistical analysis

Clontech provides an Excel spreadsheet to determine an InternallyNormalized Ratio (INR). The aim of the INR is to control for differences in labeling efficiency. An INR is calculated for each antibody antigen pair on the microarray.
The INR is calculated as follows ($Ctrl$ = spot of vehicle-treated control, Exp = spot of treated experiment):

$$Cy5 = Cy5\ mean\ intensity - Cy5\ background$$
$$Cy3 = Cy3\ mean\ intensity - Cy3\ background$$

$$Ratio\ 1 = \frac{Cy5(Ctrl{:}\ Slide\ 1, technial\ repliate\ 1)\ \times Cy3(Ctrl{:}\ Slide\ 2, technial\ repliate\ 1)}{Cy3(Exp{:}\ Slide\ 1, technial\ repliate\ 1)\ \times Cy5(Exp{:}\ Slide\ 2, technial\ repliate\ 1)}$$

$$Ratio\ 2 = \frac{Cy5(Ctrl{:}\ Slide\ 1, technial\ repliate\ 2)\ \times Cy3(Ctrl{:}\ Slide\ 2, technial\ repliate\ 2)}{Cy3(Exp{:}\ Slide\ 1, technial\ repliate\ 2)\ \times Cy5(Exp{:}\ Slide\ 2, technial\ repliate\ 2)}$$

$$\mathrm{INR} = \sqrt{\frac{Ratio\ 1 + Ratio\ 2}{2}}$$

This method relies on the background subtraction method of Genepix and it does not remove dye-bias at higher and lower intensities through normalization. Therefore, it was decided to use Limma with the LimmaGUI interface for statistical analyses (215, 216). Background correction was done via log-linear interpolation(225, 226). Spot quality weighting was performed and Genepix Flag weightings that were flagged as "Bad" were excluded from further analysis. Normalization within arrays was performed to remove dye-bias at higher and lower intensities by normalizing M-values (log-ratios) with the Global Loess method (217). Aquantile normalization between arrays was used (217).

The *M-value* (M) represents a $\log_2$-fold change between the Cy5 and Cy3 channel intensities:

$$M = Log_2(\frac{Cy5}{Cy3})$$

where$(\frac{Cy5}{Cy3})$ are the normalized emission intensities of a spot.

The Average *M-value* of a particular protein was calculated as follows:

$$M_{Technical\ replicate\ 1} = Log_2\,(\frac{Cy5(Ctrl: Slide\ 1, technial\ repliate\ 1)\ \times Cy3(Ctrl: Slide\ 2, technial\ repliate\ 1)}{Cy3(Exp: Slide\ 1, technial\ repliate\ 1)\ \times Cy5(Exp: Slide\ 2, technial\ repliate\ 1)})$$

$$M_{Technical\ replicate\ 2} = Log_2\left(\frac{Cy5(Ctrl: Slide\ 1, technial\ repliate\ 2)\ \times Cy3(Ctrl: Slide\ 2, technial\ repliate\ 2)}{Cy3(Exp: Slide\ 1, technial\ repliate\ 2)\ \times Cy5(Exp: Slide\ 2, technial\ repliate\ 2)}\right)$$

Average M

$$Average\ M = \frac{M_{Technical\ replicate\ 1} + M_{Technical\ replicate\ 1}}{2}$$

The standard deviation of $M_{Technical\ replicate\ 1}$ and $M_{Technical\ replicate\ 2}$ was calculated and proteins that had a standard deviation of less that 5% of $Average\ M$, or has an $Average\ M$ of greater than ±0.2 were considered statistically differentially expressed and were included in further analyses.

IX) Protein expression analysis

GeneVenn was used to find common genes that were affected by the compoundsbetween the MCF-7 and MDA-MB-231 cells (220).

Protein expression analysis: Caspase 3

Procaspase 3 is cleaved by executioner caspases-8, -9 and -10 into active caspase 3 (227). Active caspase 3 is an effector caspase that cleave a number of substrates resulting in morphological and biochemical features of apoptosis (227). Flow cytometry was employed to study protein expression changes of active caspase 3 in the MCF-12A, MCF-7 and MDA-MB-231 cell lines 24 h treatment of selected compounds.

Materials

A rabbit antibody for anti-active caspase 3 from IMGENEX (San Diego, California, USA) and an anti-rabbit antibody conjugated to Dylight™ 488 from Rockland Inc (Gilbertsville, Pennsylvania, USA)were purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa). The Fixation buffer, Permeabilization buffer and Assay buffer were from the FlowCellect™ Bcl-2 Activation Dual Detection Kit from Millipore Corporation (Billerica, Massachusetts, USA) that was purchased through Microsep (Pty) Ltd (Johannesburg, Gauteng, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at 1.5×10^6 cells per $25 cm^2$flask. After 24 h attachment the medium was discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h. After the allotted time of treatment, cells were trypsinized and 500 000 cells were centrifuged to discard the media. The cells were suspended in wash buffer and centrifuged again. The supernatant was removed and the cells were suspended in Fixation buffer (0.1% formaldehyde) and incubated for 20 min at room temperature. Afterwards the cells were centrifuged and the supernatant was removed. The cells were suspended in Assay buffer (1% Bovine Serum Albumin) and centrifuged. Ice cold Permeabilization buffer (500µL 100%

methanol) was added and the cells were suspended and incubated on ice for 10 min. Afterwards the cells were spun down and washed by suspending the cells in Assay buffer. The cells were spun down and suspended in 100 µL of Assay buffer with a 1:100 dilution of the primary antibody, rabbit anti-active caspase 3. The solution was incubated for 90 min on ice, after which 900 µL of Assay buffer was added to wash the cells. The cells were centrifuged and washed twice with 500 µL Assay buffer. The cells were spun down and then suspended in 100 µL of Assay buffer and 0.2 µg/mL of the an anti-rabbit antibody conjugated with the Dylight™ 488 fluoreochrome. The samples were incubated for 1 hr in the dark on ice. Afterwards the 900 µL of Assay buffer was added to wash the cells. The cells were centrifuged and washed twice with 500 µL Assay buffer. The cells were spun down and then suspended in 500 µL of Assay buffer. Fluorescence of the FL1 channel was measured with a fluorescence activated cell sorting (FACS) FC500 measured with a fluorescence activated cell sorting (FACS) FC500System flow cytometer (Beckman Coulter SA (Pty) Ltd.).Data from at least 10 000 cells were analyzed with Cyflogic (CyFlo Ltd, Turku, Finland).

Protein expression analysis: Caspase 7

Procaspase 7 is cleaved by executioner caspases-8, -9 and -10 as well as caspase 4 and 12 into active caspase 7 (227, 228). Active caspase 7 is also an effector caspase that cleave a number of substrates resulting in morphological and biochemical features of apoptosis (227). MCF-7 cellsare known to be caspase-3 deficient, since they do not express theCASP-3 gene as a result of a 47-base pair deletion within exon 3 ofthe gene, thus causing abrogated translation of CASP-3 mRNA(229). It has been discovered that mice lacking either caspase 3 or caspase 7 have a normal life span while mice without caspase 3 or -7 die shortly after birth(230, 231). This suggests that there is functional redundancybetween caspase-3 and -7 during embryogenesis and possibly in other differentiated cells, including cancer cells. Therefore it is possible for MCF-7 to undergo apoptotic cell death via caspase activation even if MCF-7 cells lack caspase 3. Flow cytometry was employed to study protein expression changes of active caspase 7 in the MCF-12A, MCF-7 and MDA-MB-231 cell lines24 h treatment of selected compounds.

Materials

A rabbit antibody for anti-active caspase 7 from BioVision Inc. (Mountain View, California, USA) and an anti-rabbit antibody conjugated to Dylight™ 488 from Rockland Inc (Gilbertsville, Pennsylvania, USA)were purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa). The Fixation buffer, Permeabilization buffer and Assay buffer were from the FlowCellect™ Bcl-2 Activation Dual Detection Kit from Millipore Corporation (Billerica, Massachusetts, USA) that was purchased through Microsep (Pty) Ltd (Johannesburg, Gauteng, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at $1.5x10^6$ cells per $25cm^2$flask. After 24 h attachment the medium was be discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h. After the allotted time of treatment, cells were trypsinized and 500 000 cells were centrifuged to discard the media. The cells were suspended in wash buffer and centrifuged again. The supernatant was removed and the cells were suspended in fixation buffer (0.1% formaldehyde) and incubated for 20 min at room temperature. Afterwards the cells were centrifuged and the supernatant was removed. The cells were suspended in Assay buffer (1% Bovine Serum Albumin) and centrifuged. Ice cold permeabilization buffer (500µL 100% methanol) was added and the cells were suspended and incubated on ice for 10 min. Afterwards the cells were spun down and washed by suspending the cells in Assay buffer. The cells were spun down and suspended in 100 µL of Assay buffer with 15 µg/mL of the primary antibody, rabbit anti-active caspase 7. The solution was incubated for 90 min on ice, after which 900 µL of Assay buffer was added to wash the cells. The cells were centrifuged and washed twice with 500 µL Assay buffer. The cells were spun down and then suspended in 100 µL of Assay buffer and 0.2 µg/mL of the anti-rabbit antibody conjugated with the Dylight™ 488 fluoreochrome. The samples were incubated for 1 hr in the dark on ice. Afterwards the 900 µL of Assay buffer was added to wash the cells. The cells were centrifuged and washed twice with 500 µL Assay buffer.

The cells were spun down and then suspended in 500 µL of Assay buffer. Fluorescence of the FL1 channel was measured with a fluorescence activated cell sorting (FACS) FC500 measured with a fluorescence activated cell sorting (FACS) FC500System flow cytometer (Beckman Coulter SA (Pty) Ltd.).Data from at least 10 000 cells were analyzed with Cyflogic (CyFlo Ltd, Turku, Finland).

Protein expression analysis: Phosphorylation of Bcl-2 at Serine 70

Bcl-2 is a key regulator of mitochondrial membrane potential and mitochondrial mediated apoptosis induction. An increase in the phosphorylation of Bcl-2 at serine 70 only lead to prevention of apoptosis induction while an increase in the multi-site phosphorylation status of Bcl-2 at serine 70, tryptophan 69 and serine 87 lead apoptosis induction (232). Multi-site phosphorylation of Bcl-2 is associated with a G_2/M block in MCF-7 and MDA-MB-231 cells (232). Dephosphorylation of Bcl-2 at serine 70 is also associated with apoptosis (232). Also, an overall decrease in the protein expression of Bcl-2 is pro-apoptotic (232). Flow cytometry was employed to study the phosphorylation status of Bcl-2 at serine 70 as well as the overall Bcl-2 protein expression in the MCF-12A, MCF-7 and MDA-MB-231 cell lines24 h treatment of selected compounds.

JNK and p38 MAP kinase inhibitor s SP600125 and SB239063 were used to gain gain further mechanistic insight into the effects that stress activated protein kinases have Bcl-2 phosphorylation.

Materials

The FlowCellect™ Bcl-2 Activation Dual Detection Kit from Millipore Corporation (Billerica, Massachusetts, USA) was purchased through Microsep (Pty) Ltd (Johannesburg, Gauteng, South Africa).

Methods

Exponentially growing MCF-12A, MCF-7 and MDA-MB-231cells were seeded at $1.5x10^6$ cells per $25cm^2$flask. After 24 h attachment the medium was be discarded and the cells exposed to the GI_{50} concentrations of the newly synthesized compounds and incubated for 24 h. After the allotted time of treatment, cells were trypsinized and 500 000 cells were centrifuged to discard the media. The cells were suspended in wash buffer and centrifuged again. The supernatant was removed and the cells were suspended in fixation buffer (0.1% formaldehyde) and incubated for 20 min at room temperature. Afterwards the cells were centrifuged and the supernatant was removed. The cells were suspended in Assay buffer (1% Bovine Serum Albumin) and centrifuged. Ice cold permeabilization buffer (500µL 100% methanol) was added and the cells were suspended and incubated on ice for 10 min. Afterwards the cells were spun down and washed by suspending the cells in Assy buffer. The cells were spun down and suspended in 100 µL of Assay buffer with an anti-Bcl-2 antibody conjugated to AlexaFluor® 488 and an anti pBcl-2 (ser70) conjugated to Phycoerythrin. The solution was incubated for 60 min on ice in the dark, after which 900 µL of Assay buffer was added to wash the cells. The cells were centrifuged and washed twice with 500 µL Assay buffer. Afterwards the 900 µL of Assay buffer was added to wash the cells. The cells were centrifuged and washed twice with 500 µL Assay buffer. The cells were spun down and then suspended in 500 µL of Assay buffer. Fluorescence of the FL1 (for measuring the Bcl-2 antibody) and FL3 (for measuring the pBcl-2, Ser 70) channel were measured with a fluorescence activated cell sorting (FACS) FC500 measured with a fluorescence activated cell sorting (FACS) FC500System flow cytometer (Beckman Coulter SA (Pty) Ltd.).Data from at least 10 000 cells were analyzed with Cyflogic (CyFlo Ltd, Turku, Finland).

Ligand-protein binding analysis: Carbonic anhydrase II and IX

X-ray crystallography and ligand binding assays of the active compounds on CAII and a mimic of CAIX were conducted in collaboration with Dr. Katherine Sippel of the McKenna Lab,

(Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Florida, USA).

Methods

The inhibition of the catalyzed exchange of ^{18}O between CO_2 and water as measured by membrane-inlet mass spectrometry was used to determine the inhibition constants (K_i) of the estradiol analogs on CAII and a CAIX mimic(233). Experiments were performed at 25 °C in 0.1 M HEPES, pH7.4 and 10 mM total carbonate concentration. Inhibitor concentrations ranged up to 8 μM and the data were processed using the Henderson method for tight-binding inhibitors(234).

Statistical analysis of data

Cell growth, metabolic activity, ELISA, mitotic indices and flow cytometry

Statistical analysis of the data was done as prescribed by Dr. Steve Olorunju of the Unit for Biostatistics at the Medical Research Council. Data was obtained from 3 independent experiments with an n-value of 6 for each repeat for crystal violet and LDH assays. Obtained data was statistically analyzed for significance using the analysis of variance-single factor model followed by a two-tailed Student's *t*-test. Means are presented in bar charts, with T-bars referring to standard deviations. *p*-values, 0.05 were regarded as statistically significant and indicated by an * or number as indicated in the legends.

Microarray and bioinformatics

Microarray analysis was conducted in conjunction with Prof. Fourie Joubert of the Bioinformatics and Computational Biology Unit of UP. Prof. Fourie Joubert assisted with basic data analysis, statistical analysis and gene identification as described in the gene expression and protein expression methodology.