



***In silico* design, synthesis and *in vitro* evaluation of
antimitotic agents**

by

Barend Andre Stander

(25432614)

Submitted in fulfillment of part of the requirements for the degree
Philosophiae Doctor (Ph.D) degree in Human Physiology

in the Faculty of Health Sciences

University of Pretoria

Pretoria

2012

Promoter: Prof AM Joubert

Co-promoter: Prof F Joubert



Promoter:

Prof AM Joubert

Department of Physiology

University of Pretoria

Tel: +27 12 319-2246

Fax: +27 12 321-1679

E-mail address: annie.joubert@up.ac.za

Co-promoter:

Prof F Joubert

Department of Biochemistry, Bioinformatics and Computational Biology Unit

University of Pretoria

University of Pretoria

Tel: +27 12 420 5802

Fax: +27 12 420 5800

E-mail address: fourie.joubert@up.ac.za

Summary

One of the most successful group of chemotherapeutic compounds currently in clinical use for anticancer treatment are those that interfere with normal progression of mitosis through the interference of microtubule dynamics. 2-Methoxyestradiol (2ME) is an endogenous metabolite of 17β -estradiol exerting both antiangiogenic and antimitogenic effects *in vitro* and *in vivo*. Abrogation of microtubule dynamics is one of the mechanisms of action of 2ME and it is proposed that 2ME interacts with the colchicine binding site of microtubules. 2ME has a short half life and has been shown to be a target for 17β -hydroxysteroid dehydrogenase-mediated metabolism. The aim of this study was to utilize non-commercial bioinformatics software to identify compounds with improved bioavailability and potency.

Academically available bioinformatics software was used to develop an *in silico* protocol in order to identify potential inhibitors of microtubule dynamics that are capable of selectively inhibiting carbonic anhydrase IX (CAIX) activity. Over expression of CAIX contributes to the acidification of the extracellular microenvironment. Acidic extracellular pH in turn contributes to the breakdown of the basement membrane, as well as the induction of the expression of proteinases that facilitate invasion and metastasis. Therefore, it was decided to identify selective inhibitors of CAIX as it provides a valuable strategy for curtailing the development of metastatic processes associated with acidotic microenvironmental conditions in tumors.

Three new and novel antimitotic compounds, ESE-15-one, ESE-15-ol and ESE-16, with nanomolar anticarbonic anhydrase activity were synthesized. Of the three new compounds, ESE-15-ol and ESE-16 were more selective towards inhibiting a mimic of CAIX over wild-type CAII. These compounds reduced cell proliferation in both the non-tumorigenic MCF-12A and the tumorigenic MCF-7 cell line in a dose-dependent manner. The compounds are 5 to 20 times more potent than 2ME. The tumorigenic MCF-7 cells and metastatic MDA-MB-231 cells were more susceptible to ESE-15-ol and ESE-16 treatment when compared to the non-tumorigenic MCF-12A cells. Morphological investigations using confocal microscopy revealed that the compounds interfere with microtubule dynamics in actively dividing cells.

Flow cytometry confirmed that the compounds are antimitotic compounds since they block cells in the G₂/M phase with subsequent induction of apoptosis via mitochondrial membrane depolarization. In ESE-16-exposed cells, JNK and p38 stress-activated protein kinases are differently affected in each cell line with the JNK pathway playing an important role in mediating mitochondrial membrane depolarization in MCF-7 cells. In MDA-MB-231 and MCF-12A cells, the p38 pathway plays a role in inducing apoptosis. Gene and proteins expression studies provided evidence for the selectivity. In MCF-7 cells, DAB2 up regulation is a likely candidate for contributing towards activating the JNK stress pathway and subsequent Bcl-2 phosphorylation and apoptosis induction. Reactive oxygen species induction due to ESE-16 exposure is argued to play an important role in inducing cell death.

Several testable hypotheses regarding the mechanism of action of ESE-16 were generated from the data. These include focusing on polyamine metabolism and its causal role in inducing apoptosis, the role that PLK2 up regulation plays in inducing apoptosis and autophagy, the role that the UPR plays in inducing cell death and lastly, the phosphorylation status of Mcl-1 in response to ESE-16 exposure. Finally, additional targets that may be used for combination treatment were identified. Altogether, the study provides a basis for future research projects to develop the newly synthesized compounds into clinically usable anticancer agents.

Keywords: Antimitotic, antiepileptic drug IX, apoptosis, autophagy, cell cycle arrest, Bcl-2, JNK, p38, mitochondrial membrane depolarization, flow cytometry, gene expression and protein microarray, anticancer

Acknowledgements

To my wife, Xiao Xing, and first born, Andrew, thank you for all your support and love. I hope to make it worth your while.

To my family - Thank you for your unwavering love and support. Their support has not been only moral but also financial.

I would like to sincerely thank the following people:

Professor Annie Joubert, supervisor and associate professor of the Department of Physiology. This work would not have been possible without her dedicated support, interest, professional guidance and resolute positive attitude. It has been a pleasure and an honor to work under Professor Joubert.

Professor Fourie Joubert, supervisor and head of the Bioinformatics and Computational Unit in the Department of Biochemistry, for help and guidance with bioinformatics.

Sumari Marais, technical assistant at the department of Physiology, for making the lab such an enjoyable environment to work in and always willing to help and provide a few words of wisdom and encouragement.

Professor Dirk van Papendorp, head of the Department of Physiology, for allowing me the opportunity to make use of the facilities at the department for this study.

Professor Dave Berger, from the Department of Botany Forestry and Agriculture Biotechnology Institute, for allowing me to make use of the ACGT Microarray Facilities.

Nicky Olivier, from the ACGT Microarray facility, for his help and guidance during the use of the facilities at the ACGT Microarray facility.

Francina and Ezekiel for their help and excellent maintenance of the laboratory.

The Cancer Association of South Africa and the National Research Foundation for providing financial support.

Friends and relatives for always being there.

I wish to acknowledge God almighty. Without Him, nothing is possible.

PUBLICATIONS

Stander BA, Marais S, Steynberg TJ, Theron D, Joubert F, Albrecht C, et al. Influence of *Sutherlandia frutescens* extracts on cell numbers, morphology and gene expression in MCF-7 cells. J Ethnopharmacol. 2007 Jun 13;112(2):312-318.

Stander A, Marais S, Stivaktas V, Vorster C, Albrecht C, Lottering ML, et al. *In vitro* effects of *Sutherlandia frutescens* water extracts on cell numbers, morphology, cell cycle progression and cell death in a tumorigenic and a non-tumorigenic epithelial breast cell line. J Ethnopharmacol. 2009 Jul 6;124(1):45-60.

Stander BA, Marais S, Vorster CJ, Joubert AM. *In vitro* effects of 2-methoxyestradiol on morphology, cell cycle progression, cell death and gene expression changes in the tumorigenic MCF-7 breast epithelial cell line. J Steroid Biochem Mol Biol. 2010 Apr;119(3-5):149-160.

Stander A, Joubert F, Joubert A. Docking, synthesis, and *in vitro* evaluation of antimitotic estrone analogs. Chem Biol Drug Des. 2011 Mar;77(3):173-181.

Stander BA, Marais S, Huyser C, Fourie Z, Leszczynski D, Joubert AM. Effects of nonthermal mobile phone radiation on breast adenocarcinoma cells. S Afr J Sci. 2011;107(9/10):1-9

Sippel K, Stander A, Tu C, Venkatakrishnan B, H Robbins A, Agbandje-McKenna M, et al. Characterization of Carbonic Anhydrase Isozyme Specific Inhibition by Sulfamated 2-Ethylestra Compounds. Letters in Drug Design and Discovery. 2011;8(8):678-684.

Stander XX, Stander BA, Joubert AM. *In vitro* effects of an in silico-modelled 17beta-estradiol derivative in combination with dichloroacetic acid on MCF-7 and MCF-12A cells. Cell proliferation. 2011 Dec;44(6):567-581.

Vorster C, Stander A, Joubert A. Differential signaling involved in *Sutherlandia frutescens*-induced cell death in MCF-7 and MCF-12A cells. J Ethnopharmacol. 2012 Mar 6;140(1):123-130.

Stander BA, Joubert F, Tu C, Sippel KH, McKenna R, Joubert AM. *In Vitro* evaluation of ESE-15-ol, an estradiol analogue with nanomolar antimitotic and carbonic anhydrase inhibitory activity. PLoS ONE. Second round of review 15 August 2012.

Stander BA, Joubert F, Tu C, Sippel KH, McKenna R, Joubert AM. Important signaling pathways of ESE-16, an antimitotic and anticarbonic anhydrase estradiol analog, in breast cancer cells. PLoS ONE. Under review 15 August 2012.

SPEAKER AND ORAL PRESENTATIONS

Speaker

2010 **B.A. Stander**, F. Joubert, and A.M. Joubert. *In silico* design, synthesis and *invitro* evaluation of novel antimetabolic molecules. The South African Society for Biochemistry and Molecular Biology, University of Free State, Bloemfontein, South Africa, January 2010.

Oral Presentations

2006 **B.A. Stander**, S. Marais, T.J. Steynberg, D. Theron, F. Joubert, C. Albrecht and A.M. Joubert. Influence of *Sutherlandia frutescens* extracts on cell numbers, morphology and gene expression profiles in a human breast adenocarcinoma cell line. Medical Faculty Day, University of Pretoria, Pretoria, South Africa, August 2006.

2006 **B.A. Stander**, S. Marais, T.J. Steynberg, D. Theron, F. Joubert, C. Albrecht and A.M. Joubert. Influence of *Sutherlandia frutescens* extracts on cell numbers, morphology and gene expression profiles in a human breast adenocarcinoma cell line. September 2006, Suid-Afrikaanse Akademie Vir Wetenskap en Kuns (Biologiese Wetenskappe), University of Johannesburg, South Africa (2006/09/08).

2006 **B.A. Stander**, S. Marais, T.J. Steynberg, D. Theron, F. Joubert, C. Albrecht and A.M. Joubert. Influence of *Sutherlandia frutescens* extracts on cell numbers, morphology and gene expression profiles in a human breast adenocarcinoma cell line. 34th meeting of the Physiology Society of Southern Africa, University of KwaZulu-Natal, Durban, South Africa (2006/09/26).

2007 **B.A. Stander**, F. Joubert, C. Albrecht and A.M. Joubert. *In vitro* effects of *Sutherlandia frutescens* extracts in human breast adenocarcinoma and normal breast epithelial cells. Medical Faculty Day, University of Pretoria, Pretoria, South Africa, August 2007.

2007 **B.A. Stander**, F. Joubert, C. Albrecht and A.M. Joubert. *In vitro* effects of *Sutherlandia frutescens* extracts in human breast adenocarcinoma and normal breast epithelial cells. 35th meeting of the Physiology Society of Southern Africa, University of the Witwatersrand, Johannesburg, South Africa (2007/09/09).

2007 **B.A. Stander**, S. Marais, C. Huyser, F. le R. Fourie, D. Leszczynski, A.M. Joubert. Invloed van nie-termiese 900 MHz draagbare foon straling op morfologie, metaboliese aktiwiteit, selsiklus progressie, apoptose induksie en globale geen ekspressie in normale bors epiteel- en borsadenokarsinoom selle. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie* (2007/09/28).

2007 **B.A. Stander**, F. Joubert, C. Albrecht and A.M. Joubert. Die *in vitro* effekte van *Sutherlandia frutescens* ekstrakte op selgroei en morfologie in 'n kankersellyn en 'n nie-

tumorigeniese epiteelbors sellyn. *Suid-Afrikaanse Tydskrif vir Natuurwetenskap en Tegnologie* (2007/09/28).

2008 **B.A. Stander**, F. Joubert, C. Albrecht and A.M. Joubert. Die *in vitro* effekte van *Sutherlandia frutescens* ekstrakte in menslike borsadenokarsinoom- en normale borsepiteit selle. September 2006, Suid-Afrikaanse Akademie Vir Wetenskap en Kuns (Biologiese Wetenskappe), University Pretoria, South Africa (2008/10/02).

2009 **B.A. Stander**, F. Joubert and A.M. Joubert. Die *in vitro* effekte van nuwe antimitotiese molekule in menslike borsadenokarsinoom selle. September 2006, Suid-Afrikaanse Akademie Vir Wetenskap en Kuns (Biologiese Wetenskappe), University of Potchefstroom, South Africa (2009/09/28). 1st Prize for best PhD presentation.

2009 **B.A. Stander**, F. Joubert, and A.M. Joubert. *In silico* design, synthesis and *invitro* evaluation of novel antimitotic molecules. 47th Microscopy Society of Southern Africa Annual Conference (MSSA), 8-11 December 2009, University of Kwazulu-Natal, Kwazulu Natal, South Africa.

2010 **B.A. Stander**, F. Joubert and A.M. Joubert. *In vitro* evaluation of a newly designed and novel antimitotic compound. Medical Faculty Day, University of Pretoria, Pretoria, South Africa, August 2010. 2nd Prize for PhD presentation.

2011 **B.A. Stander**, F. Joubert, and A.M. Joubert. A novel antimitotic and anticarbonic anhydrase IX compound induces apoptosis through the p38 stress pathway in metastatic breast adenocarcinoma MDA-MB-231 cells. South African Cell Death Society symposium, Two Oceans Aquarium, Cape Town.

2011 **B.A. Stander**, F. Joubert, A.M. Joubert. The signaling pathways involved in the induction of cell death in breast cell lines by a novel antimitotic and anticarbonic anhydrase IX compound. 39th meeting of the Physiology Society of Southern Africa, University of Western Cape, Western Cape, South Africa (2011/08/30)

2011 **B.A. Stander**, F. Joubert, A.M. Stander. Die induksie van sel dood in bors sellyne deur 'n nuwe koolsuur anhidrase 9 inhibitor antimitotiese molekule. *Suid-Afrikaanse Tydskrif vir Natuurwetenskappe en Tegnologie*, University of Johannesburg (2011/09/05). 1st Prize for best PhD presentation

List of Abbreviations

[¹⁸F] fluoro-2-deoxyglucose (FDG)
 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-
 2H-tetrazolium (WST-8)
 2,7-dichlorofluorescein (DCF)
 2,7-dichlorofluorescein diacetate (DCFDA)
 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-ol (ESE-15-ol)
 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-one (ESE-15-one)
 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10)16-tetraene (ESE-16)
 2-ethyl-3-O-sulphamoyl-estradiol (2EE)
 2-Methoxyestradiol (2ME)
 3-(N-morpholino)propanesulfonic acid (MOPS)
 4',6-diamidino-2-phenylindole (DAPI)
 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)
 9-hydroxy-5-(3,4,5-trimethoxyphenyl)-5,8,8a,9-tetrahydrofuro[3',4':6,7]
 naphtha [2,3-d][1,3]dioxol- 6(5ah)-one (POD)
 Acetylpolyamine oxidase (APAO)
 Activating transcription factor 2 (ATF2)
 Adenomatous polyposis coli (APC)
 African Centre for Gene Technologies (ACGT)
 American Tissue Culture Collection (ATCC)
 AMP-activated protein kinase (AMPK)
 Anaphase-promoting complex/cyclosome complex (APC/C)
 Apoptosis inducing factor (AIF)
 Apoptosis signal-regulating kinase 1-Trx (ASK1–Trx)
 Apoptotic protease activating factor (Apaf-1)
 Ascorbic acid (Vitamin C),
 Assisted Model Building with Energy Refinement (*AMBER*)
 Autodock free binding energy (AD4_c)
 Autodocktools4 (ADT)
 B-cell lymphoma (Bcl)
 Bcl-2 binding 3 component (BBC3)
 Bcl2-associated X protein transcript variant beta (BAX)
 Bcl2-interacting mediator of cell death (Bim),
 Bcl2-like 11 (BCL2L11)
 BH3-interacting domain death agonist (Bid)
 Bicinchoninic acid (BCA)
 Biotechnology Regional Innovation Centres (BRICs)
 Bovine fetal calf serum (FCS)
 Bovine serum albumin (BSA)
 Break point cluster region- c-Abelson (BCR-ABL)
 Breast cancer 1 early onset (BRCA1)
 Budding uninhibited by benzimidazoles 3 (BUB3)
 Cadherin1 (Cdh1)
 Calcium (Ca²⁺)

Carbonate (CO_3^{2-})
 Carbonic anhydrase IX (CAIX)
 Caspase activated DNase (CAD)
 Catalase (CAT)
 c-Jun-N-terminal kinase (JNK)
 Cluster of differentiation 95 (CD95)
 Computer-aided drug design (CADD)
 Copper-zinc superoxide dismutase (Cu/Zn-SOD)
 Crossing point (CP)
c-Src tyrosine kinase (CSK)
 Cytoplasmic protein tyrosine phosphatases (cPTPs)
 Cytoskeletal bubble (CB)
 Death effector domain (DED)
 Death inducing signal complex (DISC)
 Death receptor-3 (DR3)
 Death receptor-4 (DR4)
 Death receptor-5 (DR5)
 Deoxyribonucleic acid (DNA)
 Diacylglycerol kinase alpha (DGKA)
 Dimethyl sulfoxide (DMSO)
 Dithiothreitol (DTT)
 DNA-damage-inducible transcript 3 (DDIT/CHOP/GADD153)
 Double stranded RNA-activated protein kinase-like ER kinase (PERK)
 Dual specificity phosphatase 1 (DUSP1/MKP-1)
 Dulbecco's minimum essential medium eagle (D-MEM)
 Endoplasmic reticulum (ER)
 Epidermal growth factor receptor (EGFR)
 Epithelial-mesenchymal transition (EMT)
 Experimentally determined inhibition constant ($\text{exp}K_i$)
 Factor forkhead box O3 (Foxo3A)
 Fas associated death domain (FADD)
 Ferric iron (Fe^{3+})
 Ferrous iron (Fe^{2+})
 Fluorescence activated cell sorting (FACS)
 Front-scatter (FS)
 Gene Annotation Co-occurrence Discovery (GENECODIS)
 Generalized *AMBER* force field (GAFF)
 Glutaredoxins (Grx)
 Glutathione (GSH tripeptide),
 Glutathione disulfide (GSSG)
 Glutathione peroxidase (GPx)
 Glutathione S-transferases (GST)
 Glutathione S-transferases (GST)
 Graphic user interfaces (GUIs)
 Growth arrest and DNA-damage-inducible alpha (GADD45A)
 GTPase activating protein (GAP)

GTPase-activating proteins (GAPs)
 Guanine nucleotide dissociation inhibitors (GDIs)
 Guanine nucleotide exchange factors (GEFs)
 Guanine nucleotide releasing factors (GRFs)
 Harvey rat sarcoma virus (H-RAS)
 Heat shock 70kDa protein-2 (HSPA2)
 Heat shock 70kDa protein-5 (HSPA5)
 Heat shock 70kDa protein-6 (HSPA6)
 Heat shock protein 90 alpha (HSP90 α)
 Heat shock protein 90 beta (HSP90 β)
 Heme oxygenase 1 (HMOX1)
 Hsp70-Hsp90 Organizing stress-induced-phosphoprotein (HOP/STIP1)
 Human epidermal growth factor receptor 2 (HER2)
 Hydroethidine (HE)
 Hydrogen peroxide (H₂O₂)
 Hydroxyperoxyl ($\dot{\text{O}}\text{H}$)
 Hypoxia-inducible factor 1 alpha (HIF-1 α)
 Hypoxia-response element (HRE)
 Inhibition constant (K_i)
 Inhibitor of caspase activated DNase (ICAD)
 Inositol-requiring protein-1 (IRE1)
 Insulin growth factor 1 (IGF-1)
 Interleukin 10 (IL-10)
 Interleukin 6 (IL-6)
 Lactate dehydrogenase (LDH)
 MAD2 mitotic arrest deficient-like 1 (MAD2L1)
 Mammalian target of rapamycin (mTOR)
 Manganese superoxide dismutase (MnSOD)
 Microtubule-associated proteins 1A/1B light chain 3B membrane protein (LC3)
 Mitogen-activated protein kinase 14 (p38 α)
 Mitogen-activated protein kinases (MAPKs)
 Mitotic checkpoint complex (MCC)
 Mitotic checkpoint complex (MCC)
 Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT)
 mTOR complex 1 and 2 (mTORC1/2).
 Murine double minute 2 oncogene (mdm2)
 Myelocytomatosis viral oncogene (*c*-MYC)
N-[(7*S*)-1,2,3,10-tetramethoxy-9-oxo-6,7-dihydro-5H-benzo[d]heptalen-7-yl]ethanamide (LOC)
 N¹¹-Diethylnorspermine (DENSPM)
 National Cancer Institute (NCI)
 Nicotinamide adenine dinucleotide (NAD)
 Nicotinamide adenine dinucleotide (NADH)
 Non-receptor cytoplasmic protein tyrosine kinases (cPTKs)
 Normal+exponential (Normexp)
 Nuclear autoantigenic sperm protein (histone-binding) (NASP)

Nuclear factor-kappa Beta (NF- κ B)
 Open Graphics Library (OpenGL)
 p21cdc42/rac1-activated Ser/Thr kinase (PAK)
 p53 up regulated modulator of apoptosis (PUMA)
 Peroxiredoxin 1 (Prx 1)
 Peroxiredoxins (Prx)
 Peroxyl (RO₂[•])
 Phosphate and tensin homologue deleted on chromosome ten (*PTEN*)
 Phosphate buffered saline (PBS)
 Phosphatidylinositol (4,5)-bisphosphate (PIP2)
 Phosphatidylinositol 3-kinases (PI3K)
 Phosphatidylinositol-(3,4,5)-trisphosphate (PIP3)
 Phosphatidylserine (PS)
 Platelet-derived growth factor receptor (PDGFR)
 Polarization-optical differential interference contrast (PlasDIC)
 Polo-kinase 2 (PLK2)
 Polo-like kinase 1 (PLK1)
 Polyethylene glycol (PEG)
 Positron emission tomography (PET)
 Programmed cell death (PCD)
 Prolyl hydroxylase domain (PHD)
 Protein Data Bank (PDB)
 Protein kinase B (Akt/PKB)
 Protein kinase C (PKC)
 Python Molecular Viewer (PMV)
 Ras homolog family-related (Rho)
 Ras p21 protein activator (Ras-GAP)
 Ras p21 protein activator 2 (Rasa2)
 Ras-related C3 botulinum toxin substrate 1 (Rac)
 Rat sarcoma-related (Ras)
 Ras homolog family-related (Rho)
 Reactive oxygen species (ROS)
 Real-Time Cell Analyzer Single Plate (RTCA)
 Receptor protein tyrosine phosphatases (RPTP)
 Receptor-like protein tyrosine kinases (RPTKs)
 Receptor-mediated phosphoinositide-3 kinase I (PI3K I)
 Regulatory associated protein of mTOR (Raptor)
 Remodel the structure of chromatin (RSC)
 Research Collaboratory for Structural Bioinformatics (RCSB)
 Retinoblastoma protein (pRB)
 Reverse transcription quantitative polymerase chain reaction (RTq-PCR)
 Ribonuclease A (RNase A)
 Ribosomal protein S6 kinase 90kDa polypeptide 4 (RPS6KA4)
 Ribosomal protein S6 kinase, 70kda, polypeptide 1 (RPS6KB1)
 Root mean square deviation (RMSD)
 Serum and glucocorticoid induced kinase 1 (SGK1)

Side-scatter (SS)
Signal transducer and activator of transcription 3 (STAT3)
Simplified Molecular Input Line Entry System (SMILES)
Small GTPase Ras homolog enriched in brain protein (Rheb)
Spermidine/spermine acetyltransferase (SSAT)
Spermine oxidase (SMOX)
Spindle pole body component 25 (SPC25)
Src homology 2 domain containing protein (Shc)
Stathmin 1/oncoprotein 18 (STMN1)
Stress activated protein kinases (SAPKs)
Stress-activated protein kinases (SAPKs)
Superoxide- ($O_2^{\cdot -}$)
Superoxide dismutase (SOD)
Systems Biology Markup Language (SBML)
Systems Biology Workbench (SBW)
Thioredoxin reductase (Trx)
Transforming growth factor β 1 (TGF- β 1)
Transmission electron microscopy (TEM)
Tris buffered saline (TBS)
Tuberous sclerosis complex 1 (TSC1)
Tumor necrosis factor receptor-1 (TNFR-1)
Tumor necrosis factor receptor-associated factor 2 (TRAF2)
Tumor necrosis factor receptor-associated factor 6 (TRAF6)
Tumor protein p53 inducible nuclear protein 1 (TP53INP1),
Tumour protein 53 (TP53)
Unfolded protein response (UPR)
Vascular endothelial growth factor (VEGF)
Virtual screening (VS)
Visual Molecular Dynamics (VMD)
v-Raf-1 murine leukemia viral oncogene homolog 1 (raf-1)
V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src)
 α -tocopherol (Vitamin E)

Table of Contents

Graphical representation of biochemical pathways	1
1. Chapter 1	2
Introduction.....	2
What is Cancer?	3
Cancer Evolution	9
Gene-centered Model.....	11
Bioenergetic Model.....	13
Genome/Karyotype-centered Model.....	15
Current compounds targeting molecular biological properties associated with altered cell cycle progression in cancer cells: Antimitotic agents	17
Reactive oxygen species and cell signaling in cancer cells	20
Cell stress and cell death.....	28
Apoptosis	28
Autophagy.....	32
Current compounds targeting the bioenergetic alterations of cancer cells: Increase in acid formation and carbonic anhydrase IX as a target.....	37
Utilizing virtual screening/docking software to identify potential new compounds targeting molecular biological and bioenergetic alterations of cancer cells	38
2. Chapter 2.....	41
Materials and methods	41
Logistics.....	41
General <i>in silico</i> materials	41
Structure visualization	42
Docking.....	42
General <i>in silico</i> methods.....	44

Receptor and ligand selection	44
Receptor and ligand preparation	46
Docking.....	48
General <i>in vitro</i> materials.....	49
Cell lines	49
General reagents.....	49
Synthesis of novel compounds.....	50
General <i>in vitro</i> methods.....	50
Cell culture procedures	50
<i>In vitro</i> experimental procedures	52
Analytical experimental protocols	52
Spectrometry: Cell growth.....	52
Spectrophotometry: Cell viability.....	54
Light and Fluorescent microscopy: Polarization-optical differential interference contrast and Hoechst 33342, acridine orange and propidium iodide fluorescent staining.....	55
Confocal microscopy: Immunofluorescent morphological observation of tubulin architecture.....	57
Transmission electron microscopy: Morphological examination of intracellular ultrastructure	58
Flow cytometry: Cell cycle progression	59
Flow cytometry: Apoptosis detection	60
Flow cytometry: Mitochondrial membrane potential detection.....	62
Flow cytometry: Autophagy detection.....	63
Flow cytometry: Hydrogen peroxide and superoxide.....	64
Gene expression analysis: Complimentary RNA microarray.....	65
Gene expression analysis: Reverse transcription quantitative polymerase chain reaction	71

Protein expression analysis: Protein microarray	74
Protein expression analysis: Caspase 3	81
Protein expression analysis: Caspase 7	82
Protein expression analysis: Phosphorylation of Bcl-2 at Serine 70	84
Ligand-protein binding analysis: Carbonic anhydrase II and IX	85
Statistical analysis of data	86
3. Chapter 3	87
Results	87
Molecular modeling	87
Docking of CAII reference ligands with Autodock	87
Docking library of leads into CAII and a CAIX mimic	91
Docking library of leads into Tubulin	108
Synthesis of novel compounds	116
Cell growth studies	121
Spectrophotometry: Cell growth	121
Spectrophotometry: Cell cytotoxicity	130
Real-time Cell Growth Analysis: xCELLigence Real-Time Cell Analyzer	134
Morphological studies	136
Confocal microscopy: Immunofluorescent morphological observation of tubulin architecture	136
Light and Fluorescent microscopy: Polarization-optical differential interference contrast and Hoechst 33342, acridine orange and propidium iodide fluorescent staining	140
Transmission electron microscopy: Morphological examination of intracellular ultrastructure	151
Cell cycle progression	155
Cell signaling	166

Hydrogen peroxide and superoxideformation	166
Apoptosis induction	169
Mitochondrial membrane potential alterations	171
Autophagic activity	176
Gene expression analysis	178
Complimentary RNA microarray	178
Reverse transcription quantitative polymerase chain reaction.....	201
Protein expression analysis	204
Protein microarray	204
Caspase 3 and caspase 7 expression	208
Phosphorylation of Bcl-2 at Serine 70	211
Carbonic Anhydrase IX and II kinetics	226
4. Chapter 4.....	231
Discussion	231
5. Conclusion	249
6. References.....	250

List of Figures

Figure 1.1: Basic model for cancer evolution.....	10
Figure 1.2: The gene-centered model: Cellular alterations as the main drivers of carcinogenesis.....	12
Figure 1.3: Bioenergetic model of carcinogenesis.....	15
Figure 1.4: The genome/karyotype model of carcinogenesis.....	16
Figure 1.5: APC/C regulation and the spindle assembly checkpoint.....	19
Figure 1.6: Reactive oxygen species signaling pathways.....	23
Figure 1.7: Hydrogen peroxide metabolism.....	25
Figure 1.8: Intracellular ROS levels and cell signaling in normal and cancer cells.....	27
Figure 1.9: Cell stress-mediated caspase activation via extracellular ligands, mitochondria and the endoplasmic reticulum.....	32
Figure 1.10: Signaling and the regulation of autophagy.....	35
Figure 1.11: Schematic representation of the formation of autophagic vacuoles.....	36
Figure 1.12: Targeting CAIX to prevent metastasis.....	38
Figure 2.1: Receptor and ligand preparation protocol for redocking ligands from crystal structures into their original proteins.....	47
Figure 3.1: Correlation between Autodock free binding energy ($AD4_e$) and experimentally determined inhibition constant ($expK_i$) for carbonic anhydrase II.....	90
Figure 3.2: Docking of compounds 35, 48 and 50 into the colchicine binding site of tubulin.....	115
Figure 3.3: First proposed synthesis pathway for the compounds 34, 35, 48 and 50.....	118
Figure 3.4: Initial synthesis pathway as agreed upon between iThemba Pharmaceuticals and the Department of Physiology.....	119
Figure 3.5: Final synthesis pathway as agreed upon between iThemba Pharmaceuticals and the Department of Physiology.....	120
Figure 3.6: Preliminary dose-dependent studies conducted after 48 h exposure on ESE-15-one, ESE-15-ol and ESE-16.....	123
Figure 3.7: Cell growth curves over 72 h exposure for ESE-15-one on MCF-7, MDA-MB-231 and MCF-12A cells.....	126
Figure 3.8: Cell growth curves over 72 h of exposure for ESE-15-ol on MCF-7, MDA-MB-231 and MCF-12A cells.....	127
Figure 3.9: Cell growth curves over 72 h exposure for ESE-16 on MCF-7, MDA-MB-231 and MCF-12A cells.....	128
Figure 3.10: Cell numbers expressed as a percentage of cells relative to 100% control after exposure to different concentrations of ESE-15-one, ESE-15-ol and ESE-16.....	129
Figure 3.11: Cytotoxicity MCF-7, MDA-MB-231 and MCF-12A cells exposed to ESE-15-one, ESE-15-ol and ESE-16 for 24 h and 48 h.....	132

Figure 3.12: Real-time dynamic monitoring of cell adhesion and proliferation via the xCELLigence system ESE-16-treated cells.	135
Figure 3.13: Hoechst 33342 staining of MCF-7, MDA-MB-231 and MCF-12A cells at 100x magnification.	142
Figure 3.14: PlasDIC representations of Hoechst 33342 stained MCF-7, MDA-MB-231 and MCF-12A cells at 200x magnification after 24 h exposure.	143
Figure 3.15: PlasDIC representations of Hoechst 33342 stained MCF-7, MDA-MB-231 and MCF-12A cells at 200x magnification after 24 h exposure.	144
Figure 3.16: Hoechst 33342 and acridine orange-stained MCF-7 cells at 400x magnification after 24 h exposure. ...	145
Figure 3.17: Hoechst 33342 and acridine orange-stained MDA-MB-231 cells at 400x magnification after 24 h exposure.	146
Figure 3.18: Hoechst 33342 and acridine orange-stained MCF-12A cells at 400x magnification after 24 h exposure.	147
Figure 3.19: Hoechst 33342 and acridine orange-stained MCF-7 cells at 400x magnification after 48 h exposure. ..	148
Figure 3.20: Hoechst 33342 and acridine orange-stained MDA-MB-231 cells at 400x magnification after 48 h exposure.	149
Figure 3.21: Hoechst 33342 and acridine orange-stained MCF-12A cells at 400x magnification after 48 h exposure.	150
Figure 3.22: Transmission electron micrographs of MCF-7 cells.	152
Figure 3.23: Transmission electron micrographs of MDA-MB-231 cells.	153
Figure 3.24: Transmission electron micrographs of MCF-12A cells.	154
Figure 3.25: Cell cycle histograms of vehicle-treated, ESE-15-ol- and ESE-16-treated MCF-7 cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure.	157
Figure 3.26: Cell cycle histograms of vehicle-treated, ESE-15-ol- and ESE-16-treated MDA-MB-231 cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure.	158
Figure 3.27: Cell cycle histograms of vehicle-treated, ESE-15-ol- and ESE-16-treated MCF-12A cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure.	159
Figure 3.28: Comparisons between MCF-7, MDA-MB231 and MCF-12A cells of percentage of cells in sub G ₁ and G ₂ /M in ESE-15-ol- and ESE-16-treated cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure.	163
Figure 3.29: Comparisons of percentage of cells in sub G ₁ phase in vehicle-treated, ESE-15-ol treated and ESE-16-treated cells for MCF-7, MDA-MB231 and MCF-12A cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure. ...	164
Figure 3.30: Comparisons of percentage of cells in G ₂ /M phase in vehicle-treated, ESE-15-ol treated and ESE-16-treated cells for MCF-7, MDA-MB231 and MCF-12A cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure. ...	165
Figure 3.31: Relative mean fluorescence intensity of DCF in cells exposed to ESE-16 (200 nM) (FLG1).	167
Figure 3.32: Relative mean fluorescence intensity of hydroethidine in cells exposed to ESE-16 (200 nM) (FLG3). ..	168
Figure 3.33: Measurement of phosphatidylserine externalization in MCF-7, MDA-MB-231 and MCF-12A cells. ..	170
Figure 3.34: Relative fluorescence intensity for Mitocapture™(FL1 log) for ESE-15-ol-treated cells after 24 h exposure.	173

Figure 3.35: Relative fluorescence intensity for Mitocapture™(FL1 log) for ESE-16-treated cells after 24 h exposure.	174
Figure 3.36: Comparison of differences in mitochondrial membrane depolarization in MCF-7, MDA-MB-231 and MCF-12A cells exposed to ESE-16 over time (6 h – 48 h).....	175
Figure 3.37: LC-3 expression in MCF-7, MDA-MB-231 and MCF-12A cells after 24 exposure.	177
Figure 3.38: M/A plots of Loess normalized data.	180
Figure 3.39: GeneVenn diagram showing common genes affected in MCF-7, MDA-MB-231 and MCF12A cells.	181
Figure 3.40: GeneVenn diagram showing common proteins affected in MCF-7 and MDA-MB-231 cells exposed to ESE-16 for 24 h.....	205
Figure 3.41: Relative fluorescence intensity for Dylight™ 488-conjugated secondary antibodies bound to caspase 3 primary antibodies (FL1 log).	209
Figure 3.42: Relative fluorescence intensity for Dylight™ 488-conjugated secondary antibodies bound to caspase 7 primary antibodies (FL1 log).	210
Figure 3.43: Flow cytometry histogram, dot-plots of Bcl-2 (Ser 70) relative quantity (FL3-log) in vehicle-treated and ESE-15-ol-treated (50 nM) MDA-MB-231 cells after 24 h exposure.....	214
Figure 3.44: Flow cytometry histograms of total Bcl-2 content (FL1 Log) and Bcl-2 phosphorylated at Ser70 (FL3log) in MCF-7, MDA-MB-231 and MCF-12A cells after 24 h exposure.	216
Figure 3.45: Total Bcl-2 expression after 24 h for MCF-7, MDA-MB-231 and MCF-12A cells.	217
Figure 3.46: Bar-chart demonstrating the distribution of fluorescence intensity (FI) units of Bcl-2 (Ser 70) (FL3 Log) labeled MCF-7, cells after 24 h exposure to ESE-16 (200 nM).	218
Figure 3.47: Bar-chart demonstrating the distribution of fluorescence intensity (FI) units of Bcl-2 (Ser 70) (FL3 Log) labeled MDA-MB-231, cells after 24 h exposure to ESE-16 (200 nM).....	219
Figure 3.48: Bar-chart demonstrating the distribution of fluorescence intensity (FI) units of Bcl-2 (Ser 70) (FL3 Log) labeled MCF-12A, cells after 24 h exposure to ESE-16 (200 nM).	220
Figure 3.49: Comparison of differences in distribution of fluorescence intensity (FI) units of Bcl-2 (Ser 70) (FL3 Log) labeled MCF-7, MDA-MB-231 and MCF-12A cells after 24 h exposure to ESE-16 (200 nM).	221
Figure 3.50: Flow cytometry dot-plot of Bcl-2 (Ser 70) (FL3-log) relative quantity vs relative cellular complexity (SS lin) in MCF-7 cells.	222
Figure 3.51: Flow cytometry dot-plot of Bcl-2 (Ser 70) (FL3-log) relative quantity vs relative cellular complexity (SS lin) in MDA-MB-231 cells.....	223
Figure 3.52: Flow cytometry dot-plot of Bcl-2 (Ser 70) (FL3-log) relative quantity vs relative cellular complexity (SS lin) in MCF-12A cells.	224
Figure 3.53:Crystal structures and redocked poses of 2-ethylestradiol sulfamate (2EE) positioned in CAII and the CAIX mimic.....	229
Figure 3.54: Docking and kinetics data of ESE-15-ol.	230
Figure 4.1: Hypothesis for the mechanism of action of ESE-16 on MCF-7 cells.....	247
Figure 4.2: Hypothesis for the mechanism of action of ESE-16 on MDA-MB-231 and MCF-12A cells.	248

List of Tables

Table 3.1: Best docking energy of redocked CAII ligands.....	88
Table 3.2: Structures of estradiol ligands generated by modifying various constituents at position 2' and the D-ring of estrone.....	93
Table 3.3: Best docking energy of estrone analogs docked into CAII and a CAIX mimic.	96
Table 3.4: The CAIX:CAII ratio of transformed expK _i nM values.	100
Table 3.5: The compounds with the best CAIX:CAII ratios from transformed expK _i nM values after docking.	103
Table 3.6: The docking poses and root means squared deviation values of redocked CAII and CAIX mimic ligands.	106
Table 3.7: Best docked energy (kcal/mol) of estrone analogs docked into the colchicine binding.....	110
Table 3.8: Docking poses and root means squared deviation values of ligands binding to the colchicines binding site of tubulin.....	114
Table 3.9: Growth inhibitory effect of ESE-15-one, ESE-15-ol and ESE-16 after initial screening.....	124
Table 3.10: Growth inhibitory effect of ESE-15-one, ESE-15-ol and ESE-16 on MCF-7, MDA-MB-231 and MCF-12A cells.....	125
Table 3.11: Fold increase in cytotoxicity from 24 h to 48 h.....	133
Table 3.12: MCF-7 cells stained with DAPI and Alexa-488 anti-tubulin after 24 h exposure.....	137
Table 3.13: MDA-MB-231 cells stained with DAPI and Alexa-488 anti-tubulin after 24 h exposure.....	138
Table 3.14: MCF-12A cells stained with DAPI and Alexa-488 anti-tubulin after 24 h exposure.....	139
Table 3.15: Measurement of DNA content in MCF-7 cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure to vehicle-treated control, ESE-15-ol and ESE-16.....	160
Table 3.16: Measurement of DNA content in MDA-MB-231 cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure to vehicle-treated control, ESE-15-ol and ESE-16.....	161
Table 3.17: Measurement of DNA content in MCF-12A cells after 6 h, 12 h, 18 h, 24 h and 48 h exposure to vehicle-treated control, ESE-15-ol and ESE-16.....	162
Table 3.18: Common differentially expressed genes in MCF-7, MDA-MB-231 and MCF12A cells exposed to ESE-15-one (140 nM), ESE-15-ol (50 nM), and ESE-16 (200 nM) for 24 h.....	182
Table 3.19: Differentially expressed genes mapped to functional cellular pathways in MCF-7, MDA-MB-231 and MCF12A cells exposed to ESE-15-one (140 nM), ESE-15-ol (50 nM), and ESE-16 (200 nM) for 24 h.....	185
Table 3.20: Statistically significantly ($P < 0.05$) differentially expressed apoptosis-associate genes as determined by RTq-PCR in MCF-7, MDA-MB-231 and MCF12A cells exposed to ESE-16 for 24 h.....	202
Table 3.21: Statistically significantly ($P < 0.05$) differentially expressed cell-cycle-associate genes as determined by RTq-PCR in MCF-7 and MDA-MB-231 cells exposed to ESE-16 for 12 h.....	203
Table 3.22: Expression of proteins deemed statistically significantly.....	205
Table 3.23: Percentage of cells in the FI unit ranges of 0-7.5, 7.51-75 and 75.1-1000 as an indication of the quantity of Bcl-2 (Ser 70) phosphorylation per cell.....	215
Table 3.24: Carbonic anhydrase II and IX mimic inhibitory activity of estradiol analogues.....	228

Graphical representation of biochemical pathways

Graphical biochemical pathways were designed with CellDesigner 3.5.1. CellDesigner is a structured diagram editor for drawing gene-regulatory and biochemical networks (1). Networks are drawn based on the process diagram, with the graphical notation system proposed by Kitano (2003) *et al.* and are stored using the Systems Biology Markup Language (SBML) (2). Networks are able to link with simulation and other analysis packages through Systems Biology Workbench (SBW). CellDesigner was purely used for graphical purposes and no simulation or analyses were performed.