**In Vitro** Evaluation of ESE-15-ol, an Estradiol Analogue with Nanomolar Antimitotic and Carbonic Anhydrase Inhibitory Activity

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### Abstract

Antimitotic compounds are still one of the most widely used chemotherapeutic anticancer drugs in the clinic today. Given their effectiveness against cancer it is beneficial to continue enhancing these drugs. One way is to improve the bioavailability and efficacy by synthesizing derivatives that reversibly bind to carbonic anhydrase II (CAII) in red blood cells followed by a slow release into the blood circulation system. In the present study we describe the in vitro biological activity of a reduced derivative of 2-ethyl-3-O-sulphamoyl-estradiol (2EE), 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-ol (ESE-15-ol). ESE-15-ol is capable of inhibiting carbonic anhydrase activity in the nanomolar range and is selective towards a mimic of carbonic anhydrase IX when compared to the CAII isoform. Docking studies using Autodock Vina suggest that the dehydration of the D-ring plays a role towards the selectivity of ESE-15-ol to CAIX and that the binding mode of ESE-15-ol is substantially different when compared to 2EE. ESE-15-ol is able to reduce cell growth to 50% after 48 h at 50–75 nM in MCF-7, MDA-MB-231, and MCF-12A cells. The compound is the least potent against the non-tumorigenic MCF-12A cells. In vitro mechanistic studies demonstrate that the newly synthesized compound induces mitochondrial membrane depolarization, abrogates the phosphorylation status of Bcl-2 and affects gene expression of genes associated with cell death and mitosis.

### Introduction

The clinical usefulness of antimitotic compounds that interfere with microtubule dynamics via the colchicine binding site, including 2-methoxyestradiol (2ME), chalcones and combretastatins, are currently under investigation [1]. Mediocre biopharmaceutical properties such as short half-life and low bioavailability of 2ME have prompted the research and development of estradiol analogues with improved in vivo efficacy [2,3,4,5,6]. The addition of a sulfamate group at position 3 of the estradiol backbone is known to improve the bioavailability of estradiol analogues [7,8,9]. The sulfamate group allows the compounds to bind the carbonic anhydrase II (CAII) of red blood cells in a reversible manner [10]. This allows the bypass of first pass metabolism due to the slow release into the blood stream from CAII, resulting in increased bioavailability [7,9].

An increased acidic environment surrounding tumours are described to be the result of several metabolic alterations including increased glycolysis and lactate formation, and up regulation of extracellular carbonic anhydrase IX (CAIX) and carbonic anhydrase XII (CAXII) protein expression [11]. The conversion of carbon dioxide and water to carbonic acid by CAIX contributes to the acidification of the extracellular microenvironment [10,12]. The acidic extracellular environment associated with tumors in turn promotes the expression of proteinases that contribute to invasion and metastasis [13,14]. Compounds capable of selectivity inhibiting CAIX and therefore curtailing carbonic acid formation via CAIX over expression associated with many cancers is a strategy that can be employed to repress invasion and metastasis.

A previous study identified 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-one (ESE-15-one) as an antimitotic compound [2]. The present study describes the in vitro biological activity of a more potent reduced derivative of ESE-15-one, 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-ol (ESE-15-ol). The carbonic anhydrase kinetics of ESE-15-ol on wild-type CAII and a CAIX mimic (CAIX A65S N67Q) as well as docking poses of ESE-15-ol compared to its saturated D-ring derivative, 2-ethyl-3-O-sulphamoyl-estradiol are described [2EE] [15]. The in vitro effects of ESE-15-ol on cell growth, morphology, cell cycling and mitochondrial membrane potential in three different breast cell lines, including the metastatic MDA-MB-231, tumorigenic MCF-7 (estrogen receptor positive) and non-tumorigenic...
MCF-12A are described. ESE-15-ol was most potent against the MDA-MB-231 cells, therefore the effects of ESE-15-ol on Bcl-2 phosphorylation and global gene expression were analyzed in the MDA-MB-231 cell line.

The data in this paper suggest that ESE-15-ol is an antimitotic compound with potential anti-carbonic anhydrase IX activity that is more selective towards inhibiting growth in tumorigenic and metastatic MCF-7 and MDA-MB-231 cells when compared to non-tumorigenic MCF-12A cells. Furthermore, the data also suggests that ESE-15-ol is able to induce apoptosis by disrupting Bcl-2 dynamics in MDA-MB-231 cells.

Materials and Methods

Materials

Heat-inactivated fetal calf serum (FCS), sterile cell culture flasks, and plates were obtained through Sterilab Services (Kempton Park, Johannesburg, South Africa), Dulbecco's minimum essential medium Eagle (D-MEM), penicillin, streptomycin, and fungizone were purchased from Highveld Biological (Pty) Ltd. (Sandraham, South Africa), a primary anti-tubulin alpha antibody from IMGENE (Alexandria, VA, USA) (cat no. IMG-80196) was purchased from BIOCOM biotech (Pty) Ltd. (Clubview, South Africa). The Alexa Fluor_ 488, anti-mouse IgG H+L secondary antibody from Invitrogen (Carlsbad, CA, USA) (cat no. A21290) was purchased from The Scientific Group (Johannesburg, South Africa). The Mitocapture™ apoptosis detection kit from BioVision Inc. (Mountain View, California, USA) was purchased from BIOCOM biotech (Pty) Ltd. (Pretoria, Gauteng, South Africa). The FlowCollect Bcl-2 Activation Dual Detection Kit was purchased from Millipore Corporation (Billericca, Massachusetts, USA). Qiagen's RNeasy kit and RNase-free DNase were purchased from Agilent Technologies (Pty) Ltd. (Palo Alto, CA, USA). The pH of the extracellular growth medium of confluent MDA-MB-231 cells exposed to Desferoxamine (DFO), ESE-15-ol and a combination of DFO and ESE-16 for 24 h was measured in order to evaluate the ability of ESE-15-ol to prevent extracellular acidification by inhibiting CAIX activity [18]. An Orion 3 Star Benchtop pH meter from Thermo Fisher Scientific (Waltham, Massachusetts, USA) was used for the pH measurement of extracellular growth medium.

Molecular Modeling

All molecular modeling studies were performed on an Intel I7 920 running Ubuntu 9. Receptor and ligand preparation was carried out using ACD/ChemSketch, Chimera, Reduce, VEGA 2.2.0 and AutoDockTools 1.5.4 as described by Stander et al. (2011) [2,19,20,21,22]. Docking studies were carried out by Autodock Vina with a bounding box that encompassed the entire protein. Enzymatic activity was selected and the RMSD for 2EE was calculated with the crystal poses of 3EE bound to CAII (3oim) and CAIX mimic (3ol).

Antiproliferative Assays–crystal Violet

Quantiﬁcation of ﬁxed monolayer cells was spectrophotometrically determined employing crystal violet as a DNA stain.
Figure 1. Synthesis of ESE-15-ol, inhibition of carbonic anhydrase activity by ESE-15-ol and the effects of ESE-15-ol on extracellular acidity. A) Synthesis of 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-ol from 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-17-one. Reagents and conditions: (a) 2-ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-3-ol-17-one (0.11 mmol) and CeCl$_3$ (0.12 mmol) in MeOH : THF (9:2 v/v) at ambient temperature for 1hr. (b) After 1 hr, reaction was cooled to 0°C and NaBH$_4$ (0.21 mmol) was added and stirred for 2 hr. B) Reaction velocity ($R_1$) of wild-type CAII and a mimic of CAIX as determined by the catalysis of $^{18}$O exchange. Wild-type CAII $K_i = 167 \pm 19$ nM and CAIX mimic $K_i = 89 \pm 23$ nM, calculated using the Henderson method for tight-binding inhibitors. C) Changes in extracellular pH of confluent MDA-MB-231 cells treated with the CAIX inducer, DFO, and ESE-15-ol and a combination of DFO and ESE-15-ol. ESE-15-ol inhibited DFO-induced reduction in extracellular pH. * indicates a t-test $P$-value $< 0.05$ for difference between vehicle-treated control and the DFO-treated samples.

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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 [24,25]. Dose-dependent studies were carried out in order to determine the growth inhibitory effect on the various cell lines of the newly synthesized compounds. The growth inhibitory effect was calculated as described by the National Cancer Institute in order to compare the growth inhibition induced by the compounds on the various cell lines [26]. The vehicle-treated control for each cell line was normalized to 100%.

**Immunohistochemistry of Tubulin and Nucleus**

Confocal microscopy was employed to observe the effects of the new compounds on the cytoskeletal microtubule architecture of control and treated MDA-MB-231 cells. Cells were fixated with glutaraldehyde and alpha-tubulin was immunostained with anti-alpha tubulin antibodies. Anti-alpha tubulin antibodies were counter-stained with an Alexa-488 fluorescent probe and the nucleus was counter-stained with 4',6-diamidino-2-phenylindole (DAPI). Stained cells were viewed with a Zeiss 510 META confocal laser microscope and figures were generated with Zeiss’ ZEN 2009 software.

**Analysis of Cell Cycle**

Flow cytometry was employed to measure the DNA content of exposed and control cells in order to monitor the effect on cell cycle progression of MDA-MB-231 cells. 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. Data from at least 10 000 cells was captured with CXP software (Beckman Coulter South Africa (Pty) Ltd). Cell cycle distributions were calculated with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia) by assigning relative DNA content per cell to sub-G1, G1, S and G2/M fractions. Time-dependent studies were carried out at intervals of 12 h, 24 h and 48 h.

**Mitochondrial Membrane Potential Detection**

Mitochondrial membrane potential was monitored using MitoCapture™ by means of flow cytometry. MitoCapture™ is a cationic dye that accumulates and aggregates in the mitochondria of healthy cells, providing a bright red fluorescence [27]. In apoptotic cells, MitoCapture™ cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential and thus remains in the cytoplasm in its monomer form, generating a green fluorescence [27]. After the 24 h exposure to ESE-15-ol and the vehicle (DMSO 0.05%), cells were trypsinized and 500 000 cells were incubated with 1 ml of the MitoCapture™ reagent for 15 min. The green fluorescence of the dye was measured with a fluorescence activated cell sorting (FACS) FC500 System flow cytometer (Beckman Coulter SA (Pty) Ltd.) in the FL1 channel. Data from at least 10 000 cells were analyzed with WEASEL version 3.0 software (F. Battye, Walter and Eliza Hall Institute (WEHI), Melbourne, Australia).

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**Figure 2. Docking and kinetics data of ESE-15-ol.** Docking of compound 10 into a mimic of CAIX (A) and the wild-type isoform of CAIX (B) revealed that the double bond of ESE-15-ol at C15 and C16 may act as a nucleophile and interact with the electrophilic hydrogen of His 61. This interaction is posited to be specific of ESE-15-ol over 2EE and explain the isoform specificity of ESE-15-ol towards the CAIX mimic over CAII. Kinetics data (C) demonstrates that ESE-15-ol has an almost 2-fold higher affinity for the CAIX mimic when compared to the wild-type CAI. Experimental inhibition constants were determined by Sippel et al. (2011) [35]. The inhibition of the catalyzed exchange of 18O between CO2 and water as measured by membrane-inlet mass spectrometry was used to determine the experimental inhibition constants (K) of ESE-15-ol [16]. A CA IX mimic was used as characterized previously by Genis et al. 2009 [15].

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|                  | Experimental Inhibition Constants (K<sub>i</sub>) |
|------------------|-----------------------------------------------|
|                  | 2EE<sup>a</sup> | ESE-15-ol<sup>b</sup> |
| CAII             | 180 ± 10 nM    | 167 ± 19 nM           |
| CAIX mimic       | 2100 ± 220 nM  | 89 ± 23 nM            |

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Phosphorylation of Bcl-2 at Serine 70

Flow cytometry was employed to study the phosphorylation status of Bcl-2 at Ser 70, as well as the overall Bcl-2 protein expression in the MDA-MB-231 cell lines after 24 h exposure to ESE-15-ol compared to the vehicle-treated control. At 24 hours cells were trypsinized and 500,000 cells were centrifuged to discard the media. The cells were prepared as per the manufacturer's instructions. Fluorescence of the FL1 (for measuring the Bcl-2 antibody) and FL3 (for measuring the pBcl-2, Ser 70) channel were measured with a FACS FC500 System flow cytometer. Data from at least 10,000 cells were analyzed with WEASEL version 3.0 software.

Gene Expression

Agilent's Human 1A Oligonucleotide Microarray 4x44k slides with more than 41,000 60-mer oligonucleotide human genes and transcripts were employed to collect genomic information on the ESE-15-ol mechanism of action in MDA-MB-231 cells. The detailed protocol for the human DNA microarray analysis has been reported in a previous study [28]. Briefly, total RNA was extracted from the MDA-MB-231 cells incubated with or without ESE-15-ol according to Qiagen’s RNeasy kit protocol. Five micrograms of mRNA from each biological replicate was used per array and all tests were performed in triplicate. Slides were scanned with the Axon Genepix 4000B Scanner. Spotfinding was performed using Genepix Pro 6.1 (Molecular Devices Corporation, Sunnyvale, CA, USA). Statistical analysis after spotfinding was conducted using Limma with the LimmaGUI interface [29,30]. Background correction was done with the normal+exponential (Normexp) convolution model with an offset value was set to 25 [30,31]. The Least squares linear model fit method was employed and the P-values were adjusted for multiple testing utilizing the Benjamini and Hochberg's step-up method for controlling the false discovery rate [32]. Genes differentially expressed with a P-value of less than 0.05 were considered statistically significant. 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) [33] GENECODIS is a web-based tool for finding sets of biological annotations that frequently appear together and are significant in a set of genes [33]. In order to determine common genes that were affected by the ESE-15-ol in MDA-MB-231 cells and 2ME in MCF-7 cells, differentially expressed gene lists were compared utilizing GeneVenn [34].
and multiple spindle pole formation during mitosis. Abrogation of cell cycling including abnormal mitotic spindle formation completion formation. ESE-15-ol-treated cells show various degrees of MCF-7 and MCF-12A cells show no signs of abnormal cell cycle completion formation. ESE-15-ol-treated cells show various degrees of abrogated cell cycling including abnormal mitotic spindle formation and multiple spindle pole formation during mitosis.

**Results and Discussion**

**In vitro Carbonic Anhydrase Inhibition and Docking Analysis**

ESE-15-ol was synthesized (Figure 1A, Supporting Information S1 and S2) and assessed for carbonic anhydrase inhibition of the phosphatidylcholine (PC) liposome. The result showed that ESE-15-ol was the most potent inhibitor among the tested compounds. Kinetics data indicated that ESE-15-ol has an almost 2-fold higher affinity for the CAIX mimic when compared to the wild-type CAII (Figures 1B and 2). The analysis of the crystal structure of the CAIX mimic shows that the binding site of ESE-15-ol is very different from that of the wild-type CAII. The binding pose of ESE-15-ol is likely similar to that of 2EE into the CAIX mimic.

**Statistics**

Data was obtained from 3 independent experiments. For crystal violet studies an n-value of 6 was obtained for each repeat. Obtained data was statistically analyzed for significance using a two-tailed Student’s t-test. Means are presented in bar charts, with T-bars referring to standard deviations. Measurement of Mitocapture-derived fluorescence was expressed as a ratio of the value measured for vehicle-treated exposed cells (relative fluorescence).

**Figure 4. Vehicle-treated control and ESE-15-ol-treated cells stained with 4',6-diamidino-2-phenylindole and Alexa-488 anti-tubulin after 24 h exposure.** Vehicle-treated in MDA-MB-231, MCF-7 and MCF-12A cells show no signs of abnormal cell cycle completion formation. ESE-15-ol-treated cells show various degrees of abrogated cell cycling including abnormal mitotic spindle formation and multiple spindle pole formation during mitosis. doi:10.1371/journal.pone.0052205.g004

Fluorescence microscopy of MDA-MB-231, MCF-7 and MCF-12A cells stained with 4',6-diamidino-2-phenylindole and Alexa-488 anti-tubulin after 24 h exposure. Vehicle-treated in MDA-MB-231, MCF-7 and MCF-12A cells show no signs of abnormal cell cycle completion formation. ESE-15-ol-treated cells show various degrees of abrogated cell cycling including abnormal mitotic spindle formation and multiple spindle pole formation during mitosis. doi:10.1371/journal.pone.0052205.g004

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Fluorescence microscopy of MDA-MB-231, MCF-7 and MCF-12A cells stained with 4',6-diamidino-2-phenylindole and Alexa-488 anti-tubulin after 24 h exposure. Vehicle-treated in MDA-MB-231, MCF-7 and MCF-12A cells show no signs of abnormal cell cycle completion formation. ESE-15-ol-treated cells show various degrees of abrogated cell cycling including abnormal mitotic spindle formation and multiple spindle pole formation during mitosis. doi:10.1371/journal.pone.0052205.g004
decrease in pH of the growth medium when compared to the DMSO control. Treatment of MDA-MB-231 cells in conjunction with ESE-15-ol (100 nM) prevented the acidification (Figure 1C), suggesting that ESE-15-ol does prevent extracellular acidification due to CAIX expression.

Antiproliferative Activity of ESE-15-ol

ESE-15-ol was screened for antiproliferative activity using crystal violet as a DNA stain as described by Berry et al. (1996) [36]. The assay was conducted on metastatic MDA-MB-231 breast cancer cells, tumorigenic MCF-7 breast cancer cells (estrogen receptor positive) and non-tumorigenic MCF-12A breast cells. ESE-15-ol reduced cell proliferation in a dose-dependent manner in all tested cell lines (Figure 3). The results indicated that ESE-15-ol has the lowest GI50 concentration for the metastatic MDA-MB-231 cells (50 nM). The data indicates that the MCF-12A cell line was the least affected of the three cell lines at 50 nM. It was therefore decided carry out all subsequent studies with the GI50 of MDA-MB-231 in order to determine the differential effects of this concentration across the different cell lines.

Morphological Effects of ESE-15-ol on Tubulin Architecture

The cytoskeletal microtubule architecture of control and treated MDA-MB-231, MCF-7, and MCF-12A cells were monitored via confocal microscopy after 24 h exposure. The formation of multiple spindle poles, as well as abnormal mitotic spindle formation during mitosis was observed in ESE-15-ol-treated (50 nM) MDA-MB-231, MCF-7, and MCF-12A cells (Figure 4). Vehicle-treated cells were unaffected (Figure 4). Abrogation of

Figure 5. Effects of ESE-15-ol on cell division over time. A) Cell cycle histograms of vehicle-treated and ESE-15-ol-treated MDA-MB-231, MCF-7 and MCF-12A cells in sub G1. B) Time-dependent change of vehicle-treated and ESE-15-ol-treated MDA-MB-231, MCF-7 and MCF-12A cells in mitosis (G2/M). A gradual increase in the sub-G1 fraction is observed over time in ESE-15-ol-treatment across all cell lines, indicating increased cell death. After 48 h, the MCF-12A cells were less affected when compared to the MDA-MB-231 and MCF-7 cells. * indicates a t-test P-value <0.05 for difference between vehicle-treated control and ESE-15-ol-treated samples. † indicates a t-test P-value <0.05 for difference between MCF-7 and MCF-12A ESE-15-ol-treated samples. ‡ indicates a t-test P-value <0.05 for difference between MCF-12A and MDA-MB-231 ESE-15-ol-treated samples.

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spindle formation during mitosis is a property of antimitotic agents, confirming that ESE-15-ol is an antimitotic compound that interferes with the microtubule dynamics in actively dividing cells.

Cell Cycle Analysis

To determine the effect that ESE-15-ol has on cell cycle progression the DNA content of cells was measured after 12 h, 24 h and 48 h (Figure 5). The sub-G1 phase is indicative of cells undergoing cell death via DNA cleavage. A statistically significant time-dependent increase in the sub-G1 fraction of ESE-15-ol-treated cells was observed in all cell lines. However statistically the MCF-12A cell line was less affected by ESE-15-ol after 48 h (45.48%) when compared to MDA-MB-231 (58.41%) and MCF-7 (67.72%) (Figure 5B). In MDA-MB-231 and MCF-7 ESE-15-ol-treated cells there was an increase in the G2/M phase cells after 12 h and 24 h. After 48 h, the cells appeared to have exited the mitotic block and entered the sub-G1 phase. These results indicate that ESE-15-ol is able to induce a mitotic cell cycle arrest followed by apoptosis. It also indicates that the non-tumorigenic MCF-12A cells are less susceptible to apoptotic cell death after 48 h when compared to the tumorigenic MCF-7 and metastatic MDA-MB-231 cells.

Mitochondrial Membrane Potential Analysis

Mitochondrial membrane potential was analyzed using MitoCapture via flow cytometry. In apoptotic cells the reagent cannot aggregate in the mitochondria due to the altered membrane potential, and remains monomeric and in the cytoplasm generating a green fluorescence [27]. There was a statistically significant increase of green fluorescence in the ESE-15-ol-treated cells across all the cell lines after 24 h exposure (Figure 6). These data suggests that apoptosis is one possible form of cell death being induced by ESE-15-ol.

Phosphorylation of Bcl-2 at Serine 70

The FlowCellect Bcl-2 Activation Dual Detection Kit (Millipore) uses two antibodies to measure the abundance of Bcl-2 protein expression generally and the abundance of Bcl-2 phosphorylated at Ser 70 specifically. Our studies indicate that overall Bcl-2 expression is unchanged in ESE-15-ol-treated MDA-MB-231 cells compared to the vehicle control (data not shown). However, results indicate that the phosphorylation status of Bcl-2 in ESE-15-ol-treated MDA-MB-231 cells is very different. There were fewer cells in the fluorescence intensity (FI) unit range of 7.51–100 in ESE-15-ol-treated cells when compared to the vehicle-treated cells (Figures 7A and 7D). The FI unit range of 7.51–100 corresponds to phosphorylation status of Bcl-2 at Ser 70.
for >90% of cells in the vehicle-treated sample while it was only ±50% for the vehicle-treated cells (Figures 7A and 7D). In ESE-15-ol-treated cells the population shifted towards either an increase or decrease in phosphorylation (Figures 7A and 7D). Further analyses of the dot-plot data indicated that ESE-15-ol-treated cells with increased Bcl-2 phosphorylation tended to have a higher side-scatter (SS Lin) signal (Figures 7B, 7C and 7E). Side scatter depends on the inner complexity of the particle. For example, an increase in the amount of DNA in a cell would correlate to an increased side-scatter signal. The data suggests that ESE-15-ol-treated cells with increased inner complexity also have an increase in Bcl-2 Ser 70 phosphorylation. Bcl-2 is a key regulator of mitochondrial membrane potential and mitochondrial mediated apoptosis induction. An increase in the phosphorylation of Bcl-2 on Ser 70 alone prevents apoptosis while multi-site phosphorylation at residues Ser 70, Trp 69 and Ser 87 is associated with G2/M block in MCF-7 and MDA-MB-231 cells and leads to apoptotic induction [37]. Dephosphorylation at Ser 70 or an overall decrease in the protein expression of Bcl-2 is also associated with apoptosis [37]. These results suggest that ESE-15-ol is able to alter the balance of Bcl-2 phosphorylation in a manner that promotes apoptosis via intrinsic pathways.

Gene Expression Analysis

Agilent’s Human 1A Oligonucleotide Microarray was employed to collect transcriptional information to determine ESE-15-ol’s mechanism in MDA-MB-231 cells. Genes that were considered statistically significantly differentially expressed (adjusted P-value <0.05) and up regulated or down regulated in ESE-15-ol-treated MDA-MB-231 cells are summarized in Supporting Information S4. The 399 genes that were differentially expressed in ESE-15-ol-treated MDA-MB-MB231 cells were compared to 775 genes differentially expressed in 2ME-treated MCF-7 cells [28,34]. The differentially expressed genes were mapped to genes associated with apoptosis, autophagy, metastasis, cell cycle and stress response proteins. They include kinases, phosphatases, epigenetic and chromatin modifiers, structural proteins, transcription factors and nuclear proteins, RAS and RAB related proteins, and proteosomal components (Supporting Information S4 and S5). A total of 113 genes were found to be differentially expressed in both ESE-15-ol-treated MDA-MB-231 cells and 2ME-treated MCF-7 cells. These results indicate that there is likely a common mechanism of action between the two compounds.

Of particular interest are those genes responsive to oxidative stress including heme oxygenase (decycling) 1, spermine oxidase,
and the Bcl-2 binding 3 component (BBC3/PUMA) protein and stress-related proteins. Heme oxygenase mRNA expression is increased by the generation of reactive oxygen species such as hydrogen peroxide [30]. Spermine oxidase upregulation is known to result in antiproliferative effects produced from the oxidative stress of spermine to spermidine conversion [39,40]. An altered redox status also leads to the expression of the pro-apoptotic BBC3/PUMA protein [41]. These results suggest that the ESE-15-ol cytotoxic effect may be driven by mitochondrial mediated apoptotic induction linked to the redox status of the cell.

Additionally the transcripts differentially expressed in both 2ME- and ESE-15-ol-treated cells included cell cycle related genes and several histone cluster H3 proteins. Previous studies demonstrated that mitotic checkpoint proteins play an important role in regulating chromatin remodeling and vice versa [42,43]. Histone cluster H3 proteins help regulate the mitotic checkpoint via the tension sensing mechanism of the spindle assembly checkpoint [42,43]. Also, the “remodel the structure of chromatin” (RSC) chromatin-remodeling complex and a functioning Cdc5 protein are needed for the timely exist from mitosis in actively dividing cells [44]. Cdc5 is the yeast homologue of polo-like kinase 1 (PLK1) and is interestingly down regulated in ESE-15-ol-treated cells (Supporting Information S3). This data suggests that ESE-15-ol interferes with the normal crosstalk between the chromatin remodeling and the spindle assembly checkpoint apparatus. Further studies are needed to determine the exact mechanism of action and the implications of this interference.

Conclusions

In the present study, we performed the synthesis and in vitro biological evaluation of 2-ethyl-3-O-sulphamoyl-estr-1,3,5(10)-estr-15-tetraen-17-ol, a new antimitotic estradiol compound. Kinetics studies indicated that ESE-15-ol is more selectively towards inhibiting a CAIX mimic than wild-type CAII. Docking studies suggest that the selectivity is due to the double bond in the D-ring that is capable of interacting with His 61 of the cancer-associated CAIX. In vitro studies with a known inducer of CAIX expression in MDA-MB-231 cells suggest that ESE-15-ol can prevent extracellular acidification because of CAIX expression, suggesting that the compound has the potential to curtail metastatic processes associated with acidic microenvironmental conditions in tumors. ESE-15-ol was able to reduce cell growth to 50% of the vehicle-treated control in the nanomolar range and was more potent against metastatic MDA-MB-231 cells and tumorigenic MCF-7 cells when compared to the non-tumorigenic MCF-12A cell line. Confocal microscopy demonstrated the antimitotic effects of ESE-15-ol on actively dividing cells. Cell cycle analyses further demonstrated that ESE-15-ol is able to block cells in the G2/M phase after 12 h exposure with cell death induced after 48 h. Apoptotic cell death through mitochondrial membrane potential depolarization via the intrinsic pathway has been confirmed by flow cytometric analyses of Mitocapture fluorescence. Disruption of Bcl-2 phosphorylation as a result of ESE-15-ol exposure is likely to play a mechanistically relevant role in mitochondrial membrane potential depolarization. Also, the up regulation in the gene expression of the pro-apoptotic Bcl-2 binding protein (BBC3/PUMA) in both 2ME and ESE-15-ol-treated cells suggest a common mechanism of action between the two antimitotic compounds. Data from gene expression analyses suggest the involvement of ROS formation in inducing cell death as well as an interference in the crosstalk between the chromatin remodeling and mitotic spindle checkpoint apparatus in ESE-15-ol-treated cells. This study indicates that ESE-15-ol is a promising antimitotic anticancer drug that warrants further investigation.

Supporting Information

Supporting Information S1 Synthesis of ESE-15-ol.

Supporting Information S2 Confirmation of purity and structure and via 1H NMR (400 MHz CDCl3) and mass spectrometry.

Supporting Information S3 Crystal structures of 2EE (white) positioned in CAII (A and C) and the CAIX mimic (B). The docking poses of 2EE into CAII (A, pink ligand) and into the CAIX mimic (B, yellow ligand) have an RMSD value of 1.332 and 1.42 respectively compared to the crystal pose. ESE-15-ol docked into CAII (C, pink ligand) show a close fit compared to the crystal pose of 2EE in CAII.

Supporting Information S4 Differentially expressed genes revealed by amplified cRNA microarray and bioinformatics analyses in MDA-MB-231 cells exposed to ESE-15-ol (24 h at 50 nM).

Supporting Information S5 Common differentially expressed genes revealed by amplified cRNA microarray and bioinformatics analyses in MDA-MB-231 cells exposed 24 hours to 50 nM ESE-15-ol or MCF-7 cells exposed to 1 µM 2ME.

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Author Contributions

Conceived and designed the experiments: BAS FJ CT KHS RM AJ. Performed the experiments: BAS CT. Analyzed the data: BAS CT. Contributed reagents/materials/analysis tools: BAS FJ CT KHS RM AJ. Wrote the paper: BAS. Contributed to the experimental design of the study: BAS FJ CT KHS RM MJ. Conducted the in silico molecular modelling studies: BAS FJ. Contributed towards the carrying out of the in vitro carbonic anhydrase II and IX kinetics: CT KHS RM. Carried out the in vitro cellular and molecular studies: BAS. Contributed towards the writing and reviewing of the article: BAS AJ KHS.

References

1. Stanton RA, Germert KM, Nettles JH, Anjew R (2011) Drugs that target dynamic microtubules: a new molecular perspective. Medicinal Research Reviews 31: 493-491.
2. Stander A, Joubert F, Joubert A (2011) Docking, synthesis, and in vitro evaluation of antimitotic estrone analogs. Chemical Biology & Drug Design 77: 173–181.
3. Tinley TL, Lefl RM, Randall-Hlubek DA, Cessac JW, Wilkens LR, et al. (2003) Methoxyestradiol and analogs as novel antiproliferative agents: analysis of three-dimensional quantitative structure-activity relationships for DNA synthesis...
inhibition and estrogen receptor binding. Molecular Pharmacology 61: 1053–1069.

5. Rao PN, Cessar JW, Tinley TL, Moobery SL (2002) Synthesis and antimitic activity of novel 2-methoxyestradiol analogs. Steroids 67: 1079–1089.

6. Jourdan F, Leese MP, Dohle W, Hamed E, Fernandes E, et al. (2010) Synthesis, Antitubulin, and Antiproliferative SAR of Analogues of 2-Methoxyestradiol-3, 17-O, O-bis-sulfamate. Journal of Medicinal Chemistry 53: 2942–2951.

7. Elger W, Schwarz S, Heiden A, Reddersen G, Schneider B (1995) Sulfamates of various estrogens are prodrugs with increased systemic and reduced hepatic estrogenicity at oral application. The Journal of Steroid Biochemistry and Molecular Biology 53: 395–403.

8. Ho Y, Purshot A, Vicker N, Newman S, Robinson J, et al. (2003) Inhibition of carbonic anhydrase IX by steroidal and non-steroidal salphamates. Biochemical and Biophysical Research Communications 305: 909-914.

9. Winum_JY, SouzaZafra A, Montero JL, Supuran CT (2005) Sulfamates and their therapeutic potential. Medicinal Research Reviews 25: 186–228.

10. Pastorekova S, Ratcliffe PJ, Pastorek J (2008) Molecular mechanisms of carbonic anhydrase IX-mediated pH regulation under hypoxia. JBU Int Suppl 8: 8–15.

11. Rofstad EK, Mathiesen B, Kindem K, Galappathi K (2006) Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res 66: 6699-6707.

12. Stubb M, McSheehy PMJ, Griffiths JR, Bashford CL (2000) Causes and consequences of tumour acidity and implications for treatment. Molecular Medicine Today 6: 15–19.

13. Genis C, Sippel KH, Case N, Cao W, Avvaru BS, et al. (2009) Design of a carbonic anhydrase IX active-site mimic to screen inhibitors for possible anticancer properties. Biochemistry 48: 1322–1331.

14. Silverman DN (1982) Carbonic anhydrase: Oxygen-18 exchange catalyzed by carbonic anhydrase IX is associated with metabolic dysfunction in glutamine: using hydrogen atom contacts in the choice of side-chain amide orientation. Journal of Molecular Biology 285: 1735–1747.

15. Pedretti A, Villa L, Vistoli G (2004) VEGA–an open platform to develop chemo-and bio-informatics applications, using plug-in architecture and script programming. J Comput Aided Mol Des 18: 167–173.

16. Stander BA, Marais S, Vorster C, Joubert AM (2010) 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. The Journal of Steroid Biochemistry and Molecular Biology 119: 149–160.

17. Wettenhall JM, Sengy GK (2004) limmuGUI: a graphical user interface for linear modeling of microarray data. Bioinformatics 20: 3705-3706.

18. Ritchie ME, Silver J, Oshlack A, Holmes D, et al. (2007) A comparison of background correction methods for two-colour microarrays. Bioinformatics 23: 2700–2707.

19. Supuran CT, Scozzafava A (2007) Carbonic anhydrases as targets for medicinal chemistry. Bioorganic & Medicinal Chemistry 15: 4336-4350.

20. Pirooznia M, Nagarajan V, Vicker N, Newman S, Robinson J, et al. (2009) Genes coding interpreting gene lists through enrichment analysis and integration of diverse biological information. Nucleic acids research 37: W317.

21. Amendola R, Cervelli M, Fratini E, Polticelli F, Sallustio DE, et al. (2009) Spermine metabolism and anticancer therapy. Current Cancer Drug Targets 9: 118–130.

22. Wang Y, Casero RA Jr (2006) Mammalian polyanamine catabolism: a therapeutic target, a pathological problem, or both? Journal of Biochemistry 139: 17–25.

23. Yu J, Zhang L (2008) PUMA, a potent killer with or without p53. Oncogene 27 Suppl 1: S71–83.

24. Yao Y, Dai W (2012) Mitotic checkpoint control and chromatin remodeling. Frontiers in bioscience : a journal and virtual library 17: 976–983.

25. Letai A, Kutuk O (2008) Regulation of Bcl-2 family proteins by posttranslational modifications. Current Molecular Medicine 8: 102–118.

26. Ye CR, Tyrell RM (1989) Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UV radiation, hydrogen peroxide, and sodium arsenite. Proceedings of the National Academy of Sciences of the United States of America 86: 99–103.

27. Lu J, Xu X, Hall H, Hyland EM, Boche JD, et al. (2009) Histone h3 exerts a key function in mitotic checkpoint control. Molecular and Cellular Biology 30: 537–549.

28. Oriasa V, Calisti M, Ferrari M, Pellicioli A, Sutani T, et al. (2010) The RSC chromatin-remodeling complex influences mitotic exit and adaptation to the spindle assembly checkpoint by controlling the Cdt1 phosphatase. The Journal of cell biology 191: 981–997.