Confirming oral bioavailability of novel oestradiol analogues by liquid chromatography-tandem mass spectrometry in a murine model.

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Abstract

2-methoxyestradiol (2ME) is an endogenous 17β-oestradiol metabolite that exerts antiproliferative, antiangiogenic and antitumour activity on cancer cells both in vitro and in vivo. However, the use of 2ME as a potential anticancer agent is limited due to its poor oral bioavailability coupled to a short elimination half-life. In an attempt to improve the oral bioavailability and expand the drug targets, three sulphamoylated 2ME analogues were designed using in silico modelling and subsequently synthesized. A screening limit of 5 μg/ml for each analogue using a liquid chromatography tandem mass spectrometer (LC-MS/MS) method for 2ME analogues in serum and solvent was established. Therapeutically relevant oral bioavailability was assessed for these 2ME analogues using a murine oral absorption model (CD-1 mice) where the presence of these synthetic analogues in serum samples was assessed at two hours post dosing at 150 mg/kg of individual compounds. Blood was collected and analysed for the presence of the dosed compound and potential metabolites via LC-MS/MS. Results indicated that these analogues were present in serum above the screening limit at two hours post dosing and that there is merit to further investigation into the mode and mechanism(s) of action of ESE-15-one and ESE-15-ol and ESE-16 in vivo.

Keywords: Oral bioavailability, LC-MS/MS, 2-methoxyestradiol analogues, Murine.

Abbreviations

APC: Anaphase-Promoting Complex; CAII: Carbonic Anhydrase II; CAD: Collision Activation Dissociation Gas Flow; CXP: Collision Cell Exit Potential; CUR: Curtain Gas; Da: Dalton; DP: De-Clustering Potential; EP: Entrance Potential; ESE-15-One: 2-Ethyl-3-O-Sulphamoyl-Oestra-1, 3, 5 (10), 15-Tetraene-3-Ol-17-One; ESE-15-ol: 2-Ethyl-3-O-Sulphamoyl-Oestra-1, 3, 5 (10), 15-Tetraene-3, 17-Diol; ESE-16: 2-Ethyl-3-O-Sulphamoyl-Oestra-1, 3, 5 (10), 16-Tetraene; IC50: Half Maximal Inhibitory Concentration; HPLC: High-Performance Liquid Chromatography; TEM: Interference Temperature; kV: Kilovolts; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometer; L/h: Liters per Hour; m/z: Mass-to-Charge Ratio; M: Method; 2ME: 2-Methoxyestradiol; 2-MeOEbisMATE: 2-Methoxyoestradiol-Bis-Sulphamate; 2ME-BM: 2-Methoxyestradiol-Bis-Sulphamate; μg/ml: Microgram per Milliliter; μl: Microlitre; μm: Micrometre; mg/kg: Milligram per Kilogram; mg/ml: Milligram per Milliliter; ml: Millilitre; mm: Millimetre; min: Minutes; MRM: Multiple Reaction Monitoring; ESI-: Negative Ion Mode; SNO: Oesophageal Carcinoma Cells; PLGA: Poly-Lactic-Co-Glycolic Acid; ESI+: Positive Mode; PG: Propylene Glycol; QTOF: Quadrupole Time-of-Flight; RT: Retention Time; SRM: Selected Reaction Monitoring; THF: Tetrahydrofuran; x g: Times Gravity; V/eV: Volts per electron-Volts; v/v: Volume per Volume; W: Watt.

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Introduction

The endogenous 17β-oestradiol metabolite, 2-methoxyestradiol (2ME) that is produced by sequential hepatic hydroxylation of β-oestradiol and 2-O-methylation catalysed by cytochrome P450 enzymes and catechol-O-methyltransferase respectively, has been identified as a potential anticancer agent [1,2]. 2ME binds to the colchicine-binding site of microtubules exerting antiproliferative-, antiangiogenic- and antimitic effects both in vitro and in vivo [3-7]. Colchicine-binding site occupation by 2ME induces microtubule depolymerisation by binding to tubulin and inhibits microtubule assembly [8,9].

However, the major challenge of developing 2ME as a useful therapeutic anticancer drug is to overcome its poor oral bioavailability and undesirable pharmacokinetic properties encompassing a short elimination half-life [10]. In a phase I clinical trial using a capsule formulation of 2ME, the efficacy of the compound was reduced due to the 17-hydroxy group being targeted in 17β-hydroxysteroid dehydrogenase-mediated metabolism resulting in its rapid metabolic breakdown [11-13]. Consequently, strategies such as developing 2ME analogues, designing NanoCrystal® Dispersion techniques (such as Panzem NCD™), employing 2ME delivery systems such as hydrogel and poly-lactic-co-glycolic acid (PLGA) microspheres and producing injectable formulations were investigated in order to overcome 2ME pharmacokinetic limitations [7,8,13-17].

One particular 2ME analogue, 2-methoxyoestradiol-bis-sulphamate (2-MeOEbisMATE), has proven to be more resistant to metabolism due to sulphamoylation on positions C3 and -17, and showed enhanced oral bioavailability [15]. The increased oral bioavailability was attributed to the reversible binding of the aryl sulphamoyl group to carboxic anhydrase II (CAII) present in erythrocytes, thereby reducing the rapid hepatic first pass metabolism of the steroid analogue. With its desirable profile as a potent anticancer, antiangiogenic drug that is orally available and resistant to metabolism it has been identified as a good candidate for development as a cancer therapeutic agent which still needs to be explored.

To address the challenge of 2ME’s poor bioavailability and short half-life, Stander et al. designed three sulphamoylated 2ME analogues in silico by modifying the carbon at the C2 and -17 positions with moieties known to improve the antimitic activity and which would be expected to increase the compounds’ elimination half-life. These oestradiol analogues were specifically designed to both improve binding to tubulin, as well as to increase carboxic anhydride binding affinity. The three in silico-designed sulphamoylated 2ME analogues namely 2-ethyl-3-O-sulphamoylo-oestra-1, 3, 5 (10), 15-tetraene-3-ol-17-one (ESE-15-one), 2-ethyl-3-O-sulphamoylo-oestra-1, 3, 5 (10), 15-tetraene-3, 17-diol (ESE-15-ol) and 2-ethyl-3-O-sulphamoylo-oestra-1, 3, 5 (10), 16-tetraene (ESE-16) revealed enhanced antimitic activity in vitro [18-22]. The oestradiol analogues caused abnormal spindle morphology, disrupted intracellular microtubule integrity leading to mitotic block and consequently induced apoptosis. Generation of reactive oxygen species, an altered iron metabolism and inhibition of Bcl-2 phosphorylation were observed in vitro, which are all important for mitochondrial membrane depolarization [18-20,22]. These analogues also demonstrated binding affinity with CAII and CAIX, where ESE-15-ol and ESE-16 inhibited CAI at Nano molar concentrations, exhibiting a preferential selectivity for a mimic of CAIX [18,21,22].

This study investigated the oral bioavailability of the novel analogues and assessed whether therapeutically sufficient plasma concentrations of the compounds could be achieved using a murine oral absorption model with a new, sensitive LC-MS/MS screening method. A screening concentration limit of 5 μg/ml was determined for each of the 2ME analogues. Blood was collected from mice dosed with the individual 2ME analogues two hours (h) after oral gavage and analysed using the developed LC-MS/MS screening method for the presence of the compounds and potential related metabolites.

Materials and Methods

Logistics

Research was conducted at the Department of Physiology, School of Medicine, Faculty of Health Sciences at the University of Pretoria, Pretoria, South Africa. LC-MS/MS analysis for the investigation of screening limits was performed with an Agilent 1100 HPLC system coupled to a Sciex 4000QTrap tandem mass spectrometer (Concordia, Canada) at the Department of Pharmacology, University of Pretoria, South Africa.

Serum samples obtained from individual 2ME analogue dosed CD-1 mice, a commonly used murine model in cancer toxicology research, were analysed using a Waters® nanoACQUITY UPLC® SYNAPT® G2 high resolution mass spectrometry system and an ACQUITY UPLC BEH C18 100 × 2.1 mm, 1.7 μm column at the Department of Chemistry, University of Pretoria, South Africa. CD-1 mice were housed and maintained at the University of Pretoria Biomedical Research Centre (UPBRC), Conventional Rodent Unit, Faculty of Veterinary Sciences, University of Pretoria, and Pretoria, South Africa. CD-1 mice were sourced from Onderstepoort Biological Products SOC Ltd. (Onderstepoort, Pretoria, South Africa). Mice were group housed in Euro standard type II cages on wood shavings and received water ad libitum and irradiated animal food consisting of 22.7% protein, 5% fat, 4.5% fibre and 7.5% ash (Epol (Pty) Ltd., Pretoria, South Africa) [15,23,24]. Animals were maintained on a 12:12 h light-dark cycle at a room temperature of 22 ± 1°C to standardize behaviour and physiological effects of light intensity, wavelength and periodicity on the behaviour and physiology of the 8 mice. Mice were kept for a period of 7 d to allow for acclimatization prior to experimentation. Mice were monitored for relevant clinical signs of distress before, during and after treatment to determine their health, wellbeing and survival [25,26]. These signs specifically included rapid breathing, hunched posture, piloerection, hypoactivity, half-
closed eyes, tremors, dyspnoea, agitation, fighting and feeding ability [27]. Additional environmental variables such as humidity, air quality, ventilation and noise were minimized and standardized [28].

**Ethics statement**

The study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, and Pretoria, South Africa (402/2016) and conducted under conditions that comply with institutional committee for research ethics and integrity requirements. For the mouse study, approval was also obtained from the Animal Ethics Committee (H006-12 and H004-14) and was in compliance with the South African National Standard for the use and care of animals in scientific experiments (SANS: 10386).

**Materials**

**Drug synthesis:** The three non-commercially available *in silico*-designed sulphamoylated 2ME analogues namely ESE-15-one, ESE-15-ol and ESE-16 were synthesized by Ithemba Pharmaceuticals (Pty) Ltd. (Modderfontein, Gauteng, South Africa). The structures of these compounds are shown in Figure 1.

![Figure 1. Chemical structures of 2-methoxyoestradiol and the novel analogues. (A) 2-methoxyoestradiol (2ME); (B) 2-ethyl-3-O-sulphamoyl-oestra-1, 3, 5 (10), 15-tetraen-3-ol-17-one (ESE-15-one); (C) 2-ethyl-3-O-sulphamoyl-oestra-1, 3, 5 (10), 16 tetraene (ESE-16); (D) 2-ethyl-3-O-sulphamoyl-oestra-1, 3, 5 (10), 15-tetraen-3-ol-17-ol (ESE-15-ol) [7].](Image)

**Reagents**

All required reagents were of analytical grade and were purchased from Sigma Aldrich (St. Louis, Missouri, USA) unless otherwise specified. Acetonitrile (ultragradient ROMIL-Ultra Purity Solvent and acetonitrile 200 far UV ROMIL-Super Purity Solvent) was purchased from ROMIL (Waterbeach, Cambridge, United Kingdom). Isofluron was purchased from SAFELINE Pharmaceuticals (Pty) Ltd. (Sandton, Johannesburg, Gauteng, South Africa).

**Methods**

Screening limits using two different methods developed for both a triple quadrupole and a QTOF liquid chromatography-tandem mass spectrometer (LC-MS/MS) system were assessed and confirmed. The methods could be used to achieve the required screening limits for the analytes in available sample volumes. This was a crucial step before performing animal studies to avoid the need for repeated animal studies [29]. As the quantitation limits of these two analytical methods for all the analytes in the serum samples was confirmed to be below 5 μg/ml *in vivo*, this meant oral bioavailability studies could be performed.

**Determination of the optimal dose and bioavailability of oral administration of three novel *in silico*-designed compounds:** The dose of 150 mg/kg body weight was selected due to previous literature that reported that doses of 2ME ranging from 50 to 150 mg/kg/d were effective against tumour growth, but were not toxic to mice [6,14,30,31].

*In vivo* bioavailability studies were conducted on CD-1 mice (n=12) treated with doses of 150 mg/kg body weight considering the body weights on Table 1 [12,32]. Nine mice were treated orally with 150 mg/kg compound in propylene glycol and tetrahydrofuran (PG: THF, 9:1 v/v), while three mice were administered with excipient only as controls (PG:THF, 9:1 v/v) [15,33]. Compound administration was done using the oral gavage technique to deposit the defined amount directly into the stomach [34].

**Table 1.** Actual body weights (kg) for CD-1 mice and the dosing volume (μl) to achieve 150 mg/kg body weight.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>ESE-15-one</th>
<th>ESE-15-ol</th>
<th>ESE-16</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Body weight (kg)</td>
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<td>0.025 (83)</td>
<td>0.027 (90)</td>
<td>0.029 (98)</td>
</tr>
<tr>
<td>Dosing regimen (μl)</td>
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<td>0.025 (83)</td>
<td>0.025 (83)</td>
<td>0.024 (80)</td>
</tr>
<tr>
<td></td>
<td>0.026 (87)</td>
<td>0.025 (83)</td>
<td>0.022 (75)</td>
<td>0.024 (80)</td>
</tr>
</tbody>
</table>

**Oral treatment of animals with test compounds:** Two h following oral administration of 150 mg/kg ESE-15-one, ESE-15-ol or ESE-16, mice were subjected to deep terminal anaesthesia using isofluron and blood was collected by cardiac puncture using 26G (0.45 × 13 mm) gauge needle and 1 ml Luer syringe (Terumo Corporation, Shibuya, Tokyo, Japan) [15,33]. Blood samples from each experimental subject were transferred into anticoagulant free 5 ml tubes, allowed to clot and separated for 60 min at room temperature.

Serum was collected after centrifugation at 1800 × g at 25°C for 15 min and stored at -80°C until analysed. After a step wise extraction and protein precipitation the serum samples were analysed using a SYNAPT G2 HDMS liquid chromatography-hybrid tandem (QTOF) Mass Spectrometer system using Masslynx 4.1 software (Waters, Milford Ma, USA). Samples were screened for the presence of compound and related metabolites using a threshold concentration of 5 μg/ml.

**Serum collection for determination of screening limits of test compounds:** Ten compound naive female CD-1 mice were subjected to deep terminal anaesthesia using isofluron and blood collected by cardiac puncture using a 26G (0.45 × 13 mm) gauge needle and 1 ml Luer syringe (Terumo Corporation, Shibuya, Tokyo, Japan) [15,33]. Blood samples from each experimental subject were transferred into anticoagulant free 5 ml tubes, allowed to clot and separated for 60 min at room temperature.
mm) gauge needle and 1 ml-10 ml/Luer Solo syringe (Terumo Corporation, Shibuya, Tokyo, Japan) [33]. Blood samples from each experimental subject were placed in anticoagulant free 5 ml tubes. Approximately 0.8 ml of blood was collected from each animal. After blood was allowed to clot and separate for 60 min at room temperature, serum was separated by centrifugation at 1800 × g at 25°C for 15 min, harvested and stored in 2 ml centrifuge tubes at -80°C for screening studies.

**Sample preparation for determining screening limits of test compounds:** A stock solution concentration of 1.0 mg/ml for each compound was prepared. A Sartorius M2P microbalance (0.001 mg) was used to measure 0.148 mg of ESE-15-one, 0.130 mg of ESE15-ol and 0.140 mg of ESE-16. The excipient was prepared in a 10 ml measuring cylinder containing (PG:THF, 9:1 v/v). Each compound was dissolved in the excipient to a final concentration of 1.0 mg/ml. From the above stock solution in a dilution series and stored at -80°C for 24 h) and subsequently analysed. Excipient samples were exposed to the same two experimental storage conditions.

**Serum de-proteinisation for LC-MS/MS:** ‘Overnight’ samples were allowed to thaw at room temperature for 5 min. Both ‘overnight’ (24 h) samples and ‘same day’ samples were centrifuged at 16000 × g for 5 min using a Microfuge 16 centrifuge (Beckman Coulter, California, United States). Spiked serum (50 μl) and excipient samples (50 μl) were partitioned by a series of additions (25 μl, 25 μl and 50 μl) of acetonitrile in a 2 ml micro-centrifuge tube giving a final volume of 150 μl. Samples were vortex-mixed for 5 min between additions using a Vortex Genie 2 mixer (Model G560) and then processed in a Branson sonication bath (120 W) (Danbury, CT. USA) for 10 min. This process was repeated for every addition of acetonitrile and samples were finally centrifuged for 5 min at 16000 × g. The supernatant was transferred into a vial and dried at 40°C overnight using a CentriVap concentrator (Labconco Corporation, Kansas City, Missouri, USA).

The sample residues were reconstituted in 50 μl of 25% acetonitrile in distilled water. Prepared serum and excipient samples were analysed using an Agilent 1100 HPLC system coupled to a Sciex 4000 QTrap tandem mass spectrometer (Sciex, Concordia, Canada).

**LC-MS/MS method**

In this method, the test compounds were extracted from serum using the extraction method as described above. An injection volume of 10 μl of the reconstituted extract was analysed using the Sciex 4000QTrap LC-MS/MS system. Separation was performed using a Gemini C18 100 × 2.1 mm 3 μm column (Phenomenex). The screening method was established in both positive- (ESI+) and negative ion mode (ESI-) combined with multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) where multiple or one selected ion fragment is monitored in addition to the precursor ion respectively to ensure selectivity and sensitivity of the analytical method.

Serum and excipient samples were assayed at the Department of Pharmacology, University of Pretoria, Pretoria, South Africa using a developed and validated LC-MS/MS method for the screening of ESE-15-one, ESE15-ol or ESE-16 concentrations (M1 shows more details of the method).

**Results**

**Observations**

Mice were observed for clinical signs of toxicity in Euro standard type II cages before, during and after treatment to determine their health, well-being and any adverse effects [25,26]. Mice showed no signs of toxicity over the 2 h after dosing with the highest oral dose of 150 mg/kg.

**Screening threshold determination of animal study**

Three different blank serum samples and excipient samples (with no compound) were analysed using the developed LC-MS/MS screening method which proved to be selective for each analyte to evaluate chromatographic interference. No interferences with analyte peaks were detected when using the MRM technique.

Initial screening studies to determine threshold limits of the compounds when spiked into serum were conducted and showed that ESE-15-one, ESE-15-ol and ESE-16 could all be positively identified in serum using the MRM based LC-MS/MS screening methods at concentrations of 5 μg/ml with a signal to noise ratio of greater than 30. Figure 2 represents the peak area counts of serum extracts of these oestriadiol analogues spiked at 1.0 mg/ml each.

**Test compound stability testing**

The peak area of a compound is a measure of the concentration. The measured mean peak area of ESE-15-one ‘overnight’ samples (1.58E+03) was 28.82% lower that the area of the equivalent ESE-15-one ‘same day’ samples (2.22E+03) (Figure 2). ESE-15-one eluted from the Gemini C18 100 × 2.00 mm, 3 μm column at a retention time (RT) of 3.99 min (Figure 3). ESE-15-ol eluted at a retention time 3.89 min, with a peak area of (1.60E+04) with ‘same day’ samples, 11.87% higher than the equivalent ESE-15-one ‘same day’ samples (2.22E+03) (Figure 2). ESE-15-one eluted from the Gemini C18 100 × 2.1 mm 3 μm column (Phenomenex). The screening method was established in both positive- (ESI+) and negative ion mode (ESI-) combined with multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) where multiple or one selected ion fragment is monitored in addition to the precursor ion respectively to ensure selectivity and sensitivity of the analytical method.

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stored samples, indicating possible storage instability of the compound.

Figure 2. Percentage recovery from spiked serum for the three oestradiol analogues at 1.0 mg/ml of ‘same day’ and ‘overnight’ samples. M1 gives more details of method.

Figure 3. Selected reaction monitoring (SRM) of oestradiol analogues in solution. (A) SRM for ESE-15-one at 3.89 min for ESE-15-ol at 3.99 min and ESE-16 at 4.85 min. The insert shows focused area of image (A).

In vivo studies

Mouse serum samples (n=3) spiked with PG/THF (9:1 v/v) alone were used as control samples to assess the presence of any interferences eluting at the same retention times as the test compounds during chromatography of the serum extracts. No interferences were observed using a multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) based analysis. Figure 4 shows a total ion chromatogram (TIC) for a representative blank serum sample (serum sample from test compound naïve mouse) with a scale adjusted and superimposed TIC of serum sample spiked with 10 μg/ml of ESE-15-one, ESE-15-ol and ESE-16 and where the test compound peaks have been highlighted in black.

In vivo oral bioavailability studies revealed above threshold level concentrations of the test compounds ESE-15-one, ESE-15-ol and ESE-16 in serum samples collected 2 h after oral drug administration (Figures 5-7). Data show the TIC and selected mass spectra of serum samples from mice dosed at 150 mg/kg of ESE-15-one, ESE-15-ol or ESE-16 respectively. The mass-to-charge ratio (m/z) 355-377 and associated ionization adducts were collected for each compound. Analyses of serum samples using LC-MS/MS showed presence of the test compounds above the threshold level of 5 μg/ml for all three test compounds following oral gavage.

Discussion

Literature has shown that 2ME (2 μM) inhibited the anaphase-promoting complex (APC) in MDA-MB-435 breast cells [14]. In previous studies, the 2ME analogue, 2-MeOEbisMATE was shown to inhibit growth of human breast adenocarcinoma cell line (MDA-MB-231) with a half maximal inhibitory concentration (IC50) of 0.33 μM [35]. Additionally 2-MeOE2bisMATE, 2-methoxyestradiol-bis-sulphamate (2ME-BM) and ESE-15-one, analogues of 2ME had inhibitory effects on cell growth in the range of 0.2 μM-1 μM in several cell lines including the MCF-7 cell line and oesophageal carcinoma SNO cells, showing that the sulphamoylated derivatives of 2ME are more potent inhibitors of the cancer cell than the parent compound 2ME [19,36,37].

Since steroids have a hydrophobic scaffold, solubility is generally a concern in excipients. Theron et al. reported sulphomylation of oestradiol analogues should therefore increase their solubility in aqueous media. To determine whether the three sulphomoylated in silico-designed 2ME analogues have potential for further clinical anticancer investigation, their stability in excipient and bioavailability needed to be determined.
Research has shown that doses of 2ME ranging from 50 to 150 mg/kg/d were effective against various types of cancer in mice, without reporting serious systemic toxicity [4,14,31,38-40]. 2-MeOEbisMATE administered orally had a maximum tolerated dose of 150 mg/kg body weight [15]. In this study, 150 mg/kg body weight as a dose was therefore selected based on these previous studies where 2ME and its analogues were assessed [5,30,31].

In vivo studies in mice were conducted to assess relevant clinical signs of distress before, during and after oral administration of oestradiol analogues. Health, wellbeing and survival of the mice were therefore monitored [25,26]. No adverse effects of rapid breathing, hunched posture, piloerection, hypoactivity, half-closed eyes, tremors, dyspnoea, agitation, fighting and feeding ability were observed amongst the treated mice. Thus, in vivo studies gave no clinical indication of systemic drug toxicity [27].

Our research focuses on improving treatment systems via enhanced oral bioavailability of the in silico-designed potential anticancer drug, to enable the drug to only affect the cancer
cells and at low dosages with less frequent treatment intervals. Studies are currently being conducted using the Rapid Equilibrium Dialysis system to evaluate the binding efficacy of these sulphamoylated analogues to CAII within erythrocytes which would facilitate bypassing the first pass hepatic degradation of the steroid compounds.

**Conclusion**

To determine whether the three *in silico*-designed compounds will have potential *in vivo* anticancer therapeutic applications it was important to analyse their stability in blood and to determine the oral bioavailability *in vivo*. In order to measure the oral bioavailability, a rapid screening mass spectrometric method to identify sulphamoylated 2ME analogues in serum and solvent excipient was used. Compound stability and the ability to identify the analytes after dilution in serum was demonstrated with recovery of these compounds from ‘overnight’ stored samples that included a freeze/thaw cycle. *In vivo* studies showed therapeutically relevant concentrations of the 2ME analogues in serum samples 2 h after oral administration using a LC-MS/MS validated method. This is the first study to determine whether ESE-15-one, ESE-15-ol and ESE-16 could be detected in circulation *in vivo* after oral administration of these three different 2ME derivatives. Results from this study merit further investigation of these potential anticancer compounds *in vivo*.

**Supporting Information**

**Method 1 (M1) (Table 2)**

**Analytical column:** Gemini C18 100 × 2.1 mm, 3 μm column (Phenomenex).

**Tandem mass spectrometry conditions for assay:** Ionisation voltage: 5500; Curtain gas (CUR): 23.00; Interference temperature (TEM): 450.0; De-clustering potential (DP): 50.00; Entrance potential (EP): 10.0; TurboIonSpray nebulizer gas nitrogen (GS1): 36.00; TurboIonSpray drying gas nitrogen (GS2): 35.00; Collision activation dissociation gas flow CAD: High; Collision cell exit potential (CXP): 10.0; Collision energy: 34.

**Table 2. Gradient method of method 1.**

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**Method 2 (M2) (Table 3)**

**Analytical column:** ACQUITY UPLC BEH C18 100 × 2.1 mm, 1.7 μm column.

**Tandem mass spectrometry conditions for assay:** Mass range: Mass 50 Da to 1200 Da.

**Lock spray configuration:** Reference scan frequency (s): 10.000; Reference cone voltage (V): 40.000; Reference capillary voltage (kV): 3.100; Reference trap collision energy (kV): 2.5000; Source temperature (°C): 120; Sampling cone (V/eV) 30.000; Extraction cone (V/eV): 4.0000; De-solvation temperature (°C): 450; Cone gas flow (l/h): 10.0.

**Table 3. Gradient method of method 2.**

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Conflict of Interest

All authors declare that there are no ‘actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work’.

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