



Effects of Z-venusol, isolated from *Gunnera perpensa* L., on IL-6 and cAMP activity in human breast cancer cells *in vitro*☆



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ABSTRACT

Medicinal plants continue to play an important role in the management of many diseases, including cancer. While numerous scientific studies have reported on the effects of crude extracts, very few have isolated and investigated pure-compounds from indigenous South African plants. Isolation and studying of pure compounds extends the exploration of traditional medicines in that, once the chemical structure of the active compounds is known, changes can be made to synthesise derivatives with enhanced effectiveness and/or fewer adverse effects. This study, reports on the effects of Z-venusol, a phenylpropanoid glycoside which was isolated from an indigenous South African plant, *Gunnera perpensa* L., on interleukin-6 (IL-6) and on cyclic adenosine monophosphate (cAMP) activity in the breast cancer cells *in vitro*.

Various concentrations of pure Z-venusol, previously isolated from the roots of *G. perpensa* L., were incubated for 48 h with human breast cancer cells (MCF-7s). Conventional IL-6 and cAMP activity assays were used as per manufacturers' protocols. Drugs used as positive controls included pitovastatin (which was expected to inhibit IL-6 activity) and epinephrine and propranolol, which were expected to increase and decrease cAMP levels respectively.

The highest concentration (150 µg/mL) of Z-venusol used resulted in 51% inhibition of IL-6 activity in MCF-7s ($p < 0.01$). None of the Z-venusol concentrations, either alone or in combination with epinephrine, an agonist at adrenergic receptors, showed any statistically significant effect on cAMP levels. Surprisingly, there was a 34% elevation of cAMP levels ($p \leq 0.028$) in cells which were exposed to a combination of 150 µg/mL Z-venusol and propranolol 10 µM. We hypothesise that low β -adrenergic receptor signalling in MCF-7 cells, as reported in the literature, may explain our peculiar findings.

The previously reported apoptotic cell death, caused by Z-venusol, may be related to inhibition of IL-6. A possible role for cAMP is less clear although there may be "cross talk" between it and IL-6. More investigations are needed to confirm our findings and establish which other mediators may also be involved.

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1. Introduction

Anti-cancer drugs initially discovered from plants include vinblastine (isolated from *Catharanthus roseus* L.), etoposide (isolated from *Podophyllum peltatum* L.), paclitaxel (isolated from *Taxus brevifolia* Nutt.), topotecan and camptothecin (isolated from *Camptotheca acumenata* Decne.). Therefore, medicinal plants continue to play a role in the management of cancer.

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Cancer growth can be viewed as cell development and evolution that went astray (Komarova, 2015). Generally, there is fatal, uncontrollable proliferation and spread of the body's own malignant cells. The WHO's International Agency for Research on Cancer has reported that cancer is one of the leading causes of death worldwide with about 14.2 million new cancer cases and 8.2 million cancer deaths occurring in 2012 worldwide. Breast cancer continues to be the most common cancer in women, while lung cancer, which is the most common cancer among males, accounts for the most deaths due to any cancer type globally (GLOBOCAN, 2012). Although various combinations of drugs increase survival rates in patients with cancer, many chemotherapeutic agents are expensive and cause severe side-effects which are also costly to manage (Ubel et al., 2013; Meisenberg, 2015). Therefore, globally, there is a need for cheaper, more effective and less toxic medicines for the treatment of cancer (Kawalec et al., 2015).

Plant extracts are frequently used to investigate the *in vitro* anti-cancer (Fouche et al., 2008), antibacterial (Steenkamp and Gouws, 2006) and antifungal (Ndhala et al., 2011) effects of plants, but very

few pure active compounds have been isolated, characterized and studied (Rates, 2001). The advantages of assessing pure and characterized compounds, whose molecular structure is known are numerous. For example, the chemical structures can be altered to synthesize more pharmacologically-active derivatives. This occurred with the Nobel Prize winning discovery and isolation of an antimalarial drug called artemisinin from an indigenous Chinese plant, *Artemisia annua* L. (Normile, 2015), as well as with camptothecin, an anticancer drug (Wall et al., 1972; Oberlies and Kroll, 2004; Liu et al., 2015). Thus, recently it was reported that Z-venusol, a phenylpropanoid alkaloid pure compound, isolated from an indigenous southern African plant known as *Gunnera perpensa* L., also known as River Pumpkin, *ughobo* or *uklenza* (isiZulu language), *ipuzi lomLambo* (isiXhosa language) and *qobo* (Sesotho language), causes controlled cell-death in tumour cells, while being less toxic to normal human mammary epithelial cells *in vitro* (Mathibe et al., 2016). However, the exact molecular mechanism of action, among a multitude of possible pathways, by which Z-venusol causes controlled-cell death is not known. Therefore, in this study we investigated the effects of this compound on cyclic adenosine monophosphate (cAMP) and interleukin-6 (IL-6) activity in MCF-7 breast cancer cells *in vitro*. Cyclic adenosine monophosphate is reported to be upregulated in cancer cells, both *in vitro* and *in vivo* (Lang et al., 2004; Powe et al., 2011). We also chose cAMP because of the traditional use of *G. perpensa* in causing uterine contraction (Kaido et al., 1997; Varga and Veale, 1997). As β -adrenergic agonist action relaxes the uterus and increases cAMP, Z-venusol may possibly contract the uterus by blocking an epinephrine stimulated increase in cAMP. Also, propranolol, a β -adrenergic blocker, has been shown to improve survival in patients with breast cancer (Pasquier et al., 2011). Therefore, we expected that Z-venusol may decrease cAMP and behave like a β -adrenergic blocker since it contracts the uterus and causes apoptotic cell death in MCF-7s (Mathibe et al., 2016).

We also investigated the effects of Z-venusol on interleukin-6 (IL-6) activity in MCF-7 cells *in vitro*. Interleukin-6 is an inflammatory-associated cytokine strongly linked to different features of cancer biology like metastasis and decreased survival rates (Bachelot et al., 2003; Knüpfner and Preiss, 2010). Traditional uses of extracts of *G. perpensa* L. include inflammatory conditions such as rheumatoid arthritis and haemorrhoids.

2. Material and methods

2.1. Preparation of plant material

Isolation, purification and characterization of Z-venusol to justify that it was indeed the main active chemical constituent found in the dried roots of the *G. perpensa* L., was carried out in an assay-guided isolation as reported by Khan et al. (2004). Pure compound, in dry powder form, was supplied by Professor Siegfried Drewes, Department of Chemistry, University of KwaZulu-Natal. Stock solutions of Z-venusol powder, and all other powdered drugs used as positive controls, were prepared by dissolving them in dimethyl sulphoxide (DMSO), taking care that the final concentration of DMSO to be in contact with cells was equal or less than 0.5% (Van Tonder et al., 2014). Aliquots of all stock solutions were stored at -10°C until used. Cell specific media were used to further dilute the Z-venusol 150 $\mu\text{g}/\text{mL}$ stock solutions on the day of the experiment. The cells were exposed for 48 h to various working concentrations Z-venusol; that is, 37.5 and 75 $\mu\text{g}/\text{mL}$, which were based on unpublished pilot cell viability experiments, and at the end of the exposure time, supernatants (*i.e.*, samples) were stored at -10°C until used.

2.2. Cell line and medium

Epithelial human breast cancer cells, the Michigan Cancer Foundation-7 (MCF-7s), obtained from Highveld Biological, National

Repository of the Cancer Association of South Africa, were used. These cells were grown in DMEM (Biowhittaker, USA) supplemented with 10% foetal bovine serum (FBS), L-glutamine and Penicillin Streptomycin Fungizone® (PSF) and 0.1% sodium pyruvate, all purchased from Sigma, St. Louis, USA.

2.3. Human interleukin-6 (IL-6) activity assay

The human IL-6 sR ELISA kits (Lot # 0317C0367), purchased from Sigma, were used as per manufacturer's protocol. Briefly, all reagents were brought to room temperature. Thereafter, 100 μL of each standard and sample was added into appropriate wells coated with human IL-6 sR antibodies, covered and incubated at $37^{\circ}\text{C}/5\%\text{CO}_2$ and 100% relative humidity. On the following day, solutions were discarded and each well was washed 4 times with 300 μL of $1\times$ Wash Solution. Then, 100 μL of prepared biotinylated detection antibody solution was added to each well and incubated on the gentle shaker at room temperature for 1 h. Thereafter, the solution was discarded and each well was washed with $1\times$ Wash Solution as described above. Then, 100 μL of prepared HRP-Streptavidin solution was added to each well, and incubated with gentle shake at room temperature for 45 min. Thereafter, the solution was discarded and each well was washed with $1\times$ Wash Solution as described above. Then, 100 μL of ELISA colourimetric tetramethylbenzidine (TMB) reagent was added to each well, and incubated in the dark with gentle shake at room temperature for 30 min. Finally, 50 μL Stop Solution was added to each well and absorbance was read using the BioRad (Model 3550) microplate reader, at 595 nm wavelengths. The highest final working concentration for a drug used as a positive control, pitovastatin, was 1.5 μM (Wang and Kitajima, 2007). Three independent experiments were carried out in quadruplicate ($n = 12$) and outliers were removed.

2.4. The direct acetylated cyclic adenosine monophosphate (cAMP) assay

The direct cAMP enzyme immunoassay kits (Lot # SLBM17230), purchased from Sigma were used as per manufacturer's protocol. Briefly, all reagents were thawed to room temperature. The acetylation reagent (or solution) was prepared by adding 0.5 mL of acetic anhydride to 1 mL of triethylamine. Subsequently, 10 μL of acetylation solution was added to every 200 μL of standard and sample solutions to enhance the detection of cAMP activity, because there is a low expression of β -adrenergic receptors in MCF-7s (Madden et al., 2011). Various concentrations (0.078, 0.312, 1.25, 5 and 20 pmol/mL) of standard solution were prepared by serial dilution of cAMP solution with 0.1 M HCl. Then, 50 μL of neutralising reagent was added into each well of the 96-well-plate coated with goat anti-rabbit IgG antibodies. Thereafter, 100 μL of standard solutions and 100 μL of sample solutions added to appropriate wells. This was followed by addition of 50 μL of the blue cAMP-Alkaline Phosphatase Conjugate into appropriated wells and inoculation of 50 μL of the yellow cAMP EIA antibody solution. Then, the plate was incubated on a gentle shaker at room temperature for 2 h. Thereafter, the contents were emptied and the wells were washed three times with a freshly prepared 10% wash buffer solution. Then, 200 μL of the p-nitrophenyl phosphatase conjugate substrate solution was added to each well and thereafter the plate was incubated at room temperature, protected from light, for 1 h. Finally, 50 μL Stop Solution was added to each well and absorbance was read using the BioRad (Model 3550) microplate reader, at 415 nm wavelengths. The measured optical intensity is inversely proportional to the concentration of cAMP in either the samples or the standards. Epinephrine (10 μM) and propranolol (10 μM), both purchased from Sigma, were used separately, in combination and added to the highest concentration of Z-venusol for comparison (Kelly et al., 2009; Madden et al., 2011). Four independent experiments were carried out in duplicates ($n = 8$) and five outliers (representing 5.2% of the data), which could not fit into the standard curve were removed.

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