Novel acylated steroidal glycosides from *Caralluma tuberculata* induce caspase-dependent apoptosis in cancer cells

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Abstract

Aim of the study: Pregnane glycosides are potent cytotoxic agents which may represent new leads in the development of anti-tumour drugs, particularly in the treatment of breast cancer, because of the structural similarity to estrogenic agonists. *Caralluma* species are natural sources of a wide variety of pregnane glycosides. The aim of the study was to isolate, using an activity-guided fractionation approach, novel pregnane glycosides for testing on breast cancer and other tumour lines.

Materials and Methods: The effect of crude extracts, specific organic fractions and isolated compounds from *Caralluma tuberculata* was tested on the growth and viability of MCF-7 oestrogen-dependent, and MDA-MB-468 oestrogen-independent breast cancer cells, Caco-2 human colonic cells, HUVECs and U937 cells. Neutral red uptake and MTT assays were used. Apoptosis was detected by Western blot of poly-(ADP ribose) polymerase (PARP) as were other markers of nuclear fragmentation (DNA ladder assay, staining of cells with nuclear dye DAPI). The involvement of caspases was investigated using the pan-caspase inhibitor Z-VAD-FMK.

Results: The ethyl acetate fraction of *Caralluma tuberculata* was found to be the most potent anti-proliferative fraction against all three cancer cell lines. Two novel steroidal glycosides were isolated from the active fraction after a series of chromatographic experiments. The structure of the isolated compounds was elucidated solely based on 2D-NMR (HMBC, HETCOR, DQF-COSY) and MS spectral analysis as Compound **1:** 12-*O*-benzoyl-20-*O*-acetyl-3β,12β,14β,20β-tetrahydroxy-pregnan-3-yl*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-3-methoxy-β-D-ribopyranoside, and as Compound **2:** 7-*O*-acetyl-12-*O*-benzoyl-3β,7β,12β,14β-tetrahydroxy-17β-(3-methylbutyl-*O*-acetyl-1-yl)-androstan-3-yl*O*-β-D-glucopyranosyl-(1→4)-6-deoxy-β-D-allopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymapyranosyl-(1→4)-β-D-cymaropyranoside. Compound **1** (pregnane glycoside) and compound **2** (androstan glycoside) induced apoptosis at <25 μM after 48 hours as assessed by cell shrinkage, PARP cleavage, DNA fragmentation, and reversal with the caspase inhibitor.

Conclusions: Two novel steroid glycosides isolated from *Caralluma tuberculata* possess moderate, micromolar cytotoxic activity on breast cancer and other cells *in vitro*, which may indicate a source of activity *in vivo* of interest to future drug design.

Keywords

Caralluma tuberculata, Asclepiadaceae, Pregnane glycosides, Androstan glycosides, Breast cancer, MTT assay, Neutral Red uptake assay

1. Introduction

Caralluma tuberculata (N.E.Brown) a member of the Asclepiadaceae family, is a succulent, perennial herb growing in the wild in Pakistan and India (Andra Pradesh), United Arab Emirates, Saudia Arabia, the south east of Egypt (Tackholm et al, 1974; Baquar et al, 1989), and other countries. In the literature, the plant has also been reported as *Boucerosia aucheriana* (Ali *et al*, 1983).

The roots of *Caralluma tuberculata* are eaten raw, or cooked as a vegetable, (Ali *et al* 1983, Ahmad *et al* 1988), and the plant is traditionally used for its anti-inflammatory and anti-tumour properties (Ahmad *et al* 1983, 1988; Ahmed *et al* 1993, Copra *et al* 1956, Mahmood *et al* 2010). Several pregnane glycosides isolated from organic extracts of *Caralluma tuberculata* showed cytotoxicity against the MRC5 human diploid embryonic cell line (Abdel-Sattar *et al* 2008, 2009). Pregnanes are C_{21} steroids and often found in nature conjugated as glycosides. *Caralluma* and other members of the *Asclepiadaceae* family are rich in esterified polyhydroxypregnane glycosides, which have potentially important anti-cancer and anti-tumour effects (Deepak *et al*. 1989, 1997; Gupta *et al* 2003), and may provide interesting leads for the development of new drugs.

The aim of the present study was to isolate novel pregnane and related steroids from *Caralluma tuberculata* and to elucidate their structures and cytotoxicity. The next step was to use an activity-guided fractionation approach to identify those organic fractions of *Caralluma tuberculata* with the highest anti-proliferative activity against MCF-7 human breast cancer cells and Caco-2 human colon cells using the MTT and neutral red uptake assays. Following identification of any compounds isolated in the most active fractions, the cytotoxicity of these compounds would then be tested on the cell lines and the mode of cell death investigated using established assays of apoptosis and necrosis.

2. Materials and Methods

2.1 Plant material

Caralluma tuberculata was collected from the surrounding areas of Mansehra Valley, Pakistan in February 2007, and identified by Dr. Manzoor Hussain, Department of Botany, Hazara University, Mansehra, Pakistan. A voucher specimen (HU-2761A-07) was deposited at the Herbarium of Department of Botany, Hazara University, Mansehra, Pakistan.

2.2 Extraction and Fractionation

The whole fresh plant (10 kg) was air-dried, powdered and macerated in EtOH (3 x 1000 ml) at room temperature for 7 days. The extract was filtered under vacuum and evaporated under reduced pressure at 40^{o}C. This yielded a semi-solid residue, dark greenish in colour (200 g). The concentrated extract was dispersed in H_2O (1000 ml), partitioned into four organic fractions starting with hexane (3 x 300 ml), $CHCl_3$ (3 x 500 ml) and EtOAc (3 x 500 ml) respectively. The MeOH fraction (3 x 300 ml) was separated from the insoluble residue after evaporating the remaining aqueous layer. This procedure resulted in hexane fraction (5 g), $CHCl_3$ fraction (8 g), EtOAc fraction (20 g), MeOH fraction (22g) and aqueous fraction (28 g).

2.3 Isolation of compounds

The EtOAc fraction was loaded (12 g) on silica gel 60 (2 kg) for column (100 cm x 12 cm) adsorption chromatography packed in $CHCl_3$. Stepwise elution was carried out using $CHCl_3$-MeOH gradient solvent system (1:0, 20:1, 10:1, 8:1, 5:1, 3:1, 2:1, and 0:1; 2.0 L for each step). Fractions of 200 ml were collected and concentrated; TLC was carried out for all the eluted fractions and visualised under UV illumination (254nm). Fractions possessing similar R_f values were combined together and this resulted in 25 major fractions. Fraction 18 (352 mg) showed a mixture of two compounds on TLC. The fraction was chromatographed on a silica gel column (500 g, 3.5 cm x 60 cm) and eluted with EtOAc-MeOH (5:1, 4:1 1 L each eluent). Thin Layer Chromatography was carried out on silica gel 60 F_{254}, precoated aluminium cards (0.2 mm thickness) from Merck Ltd, Germany. Repeated adsorption column chromatography of Fraction 18 resulted in three sub-fractions. Sub-fraction I yielded Compound **1** (62 mg) while Sub-fraction II and III were further purified over RP-C18 column (100 g, 40 cm x 3 cm) with MeOH-H_2O (80:20, 0.5 L) to give Compound **2** (75 mg). Silica gel 90 C18-Reversed phase (60757) for Column Chromatography was purchased from Fluka Analytical, Switzerland. To determine the steroidal nature of the compounds, the EtOAc fraction and isolated Compounds **1** and **2** were analysed by

Liebermann-Burchard test (Burke et al., 1974; Halim & Khalil 1996). Melting points were determined on a Gallenkamp (Sanyo) melting point instrument and are uncorrected. Optical rotations were measured in MeOH solutions with a Optical Activity AA-10 automatic polarimeter. FT-IR Spectra were recorded on PerkinElmer Spectrum 100FT-IR spectrometer with Spectrum Express sodium chloride discs.

2.4 NMR and mass spectrometry (MS) analysis

^{1}H, ^{13}C-NMR, DEPT, edited DEPT and two-dimensional HETCOR, DQF-COSY, HMBC NMR spectra were recorded in methanol-D_3 (CD_3OD) using JEOL Eclipse 400 MHz NMR spectrometer with Jeol Delta version 7.2 control and processing software. Analysis was carried out on GC-MS (Agilent Technologies) 5890 GC with 5973 Mass spectrophotometer, operating with electron ionisation with Helium gas as carrier. HR Mass was recorded on WATER's LCT mass spectrophotometer with time-of-flight (TOF) using electron spray ionisation (ESI) connected to Alliance auto-sampler injection system. ESI-MS fragmentation pattern was recorded on LC MS/MS TSQ Quantum Access (Thermo Electronic Corporation UK) with ACCELA auto-sampler.

2.5 Cell culture

MCF-7 (human breast estrogen-dependent adenocarcinoma) and MDA-MB-468 (human breast estrogen-independent adenocarcinoma) cells were obtained from LGC Standards, Teddington, UK. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat inactivated fetal bovine serum (FBS) and gentamycin (40 μg/ml), penicillin (100 units/ml) and streptomycin (1040 μg/ml). The Caco-2 (human colon adenocarcinoma) cell line was obtained from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK) and grown in complete growth medium: Dulbecco's Modified Eagle's Medium (DMEM) containing 10% v/v FBS, 2 mM L-glutamine (all materials from Sigma Aldrich, UK). U937 cells were from LGC Standards (Teddington, UK) and were grown in RPMI-1640 Medium supplemented with FBS to a final concentration of 10% and gentamycin (40 μg/ml), penicillin (100 units/ml) and streptomycin (1040 μg/ml). HUVEC (human umbilical vein endothelial cells) were isolated from umbilical cords (kindly donated by Dr Anshuman Ghosh, School of Life Sciences, Kingston University, UK) by enzymatic detachment using collagenase, as previously described (Bernhard et al., 2003). HUVEC Cells were routinely passaged in 0.2% gelatine-coated (Sigma, Steinheim, Germany) polysterene culture flasks (Becton Dickinson, Meylan Cedex, France) in MCDB 131 growth media (Invitrogen Ltd. Paisley UK) supplemented with EGM Single Quots Supplements and growth factors (Invitrogen Ltd. Paisley UK).

All cell lines were grown in a humidified incubator at 37^{o}C in an atmosphere containing 5% CO_2 and were in the logarithmic phase of growth at the time of cytotoxicity assays. Cells were harvested and seeded into 96-well tissue culture plates at a density of $1x10^4$ cells per well in 200 µl aliquots of medium. The cells were allowed to attach for 24 h at 37°C, 5% CO_2 in air in a humidified atmosphere. The next day, the plant extract in serial dilution or desired final concentration of organic fraction and isolated glycosides dissolved in DMSO (maximum: 0.01%) were added to the desired final concentrations and after a 24 h exposure period, the toxic endpoints were determined. Control groups received the same amount of DMSO. Actinomycin-D (4µM), tamoxifen (5µM) and anastrozole (5µM) were used as positive controls in 200µl media as a final concentration in the well.

2.6 Neutral red assay

According to the Borenfreund & Puerner (1985) method of measuring of inhibition of cell growth, neutral red is a vital dye taken up by cells and incorporated in lysosomes. Dead or damaged cells do not take up the dye. The neutral red uptake assay was performed by removal of the medium after dosing cells and 200 µl of neutral red solution (40 µg/ml) was added to all wells (including the blanks, which contained medium only). After incubation for 2.5 hours, the neutral red was removed, cells were carefully rinsed with pre-warmed D-PBS, and 200 µl of ethanol/acetic acid (1% glacial acetic acid in 5% ethanol) was added to all wells. The plate was covered in foil and placed on a plate shaker for 30 minutes to extract neutral red from the cells and form a homogeneous solution. Absorbance of the wells was measured at 540 nm in a microplate reader (Labtech LT-4000MS, Labtech International Ltd. Acorn House, East Sussex, UK) within 60 minutes.

2.7 MTT assay

According to method described by Borenfreund et al. (1988) growth of cancer cells was quantified by the ability of mitochondrial succinic dehydrogenase enzyme in the living cells to reduce the yellow dye 3-(4,5-dimethyl- 2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue insoluble product formazan. Following a 24 h exposure period of drugs, cells were washed twice with PBS, and a 10 µl/well of MTT reagent (5 mg/ml in PBS) was added. The plates were returned to the incubator for 4 h. Subsequently, cells were washed twice with PBS, and 100 µl/well DMSO was added as solvent to dissolve the insoluble crystalline formazan products. Drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

2.8 Cytomorphological alterations (DAPI staining)

The nuclear stain DAPI was used to assess morphological changes in nuclei of treated cells. Cells were seeded at $1x10^4$cells/well in 500µl of DMEM on sterilised glass coverslips in 12 well plates for 24 h. All three cells line (MCF-7, MDA MB-468, Caco-2) were treated with negative control (culture medium), positive control (actinomycin-D 4µM) along with pregnane and androstan glycosides according to there specific IC_{50} values for particular cell line. The plates were incubated under above mentioned conditions for 24 hours. After treatment, cells were briefly equilibrated with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilised with methanol for 5 min, and mounted in a DAPI-containing medium (VectorShield, Vector Labs, Peterborough, UK). Fixed cells were visualized using a confocal fluorescence microscope (Leica TCS SP2, Milton Keynes, UK)

2.9 Detection of poly (ADP-ribose) polymerase (PARP) by Western blot.

PARP is a vital enzyme in DNA repair and the presence of its cleaved form is an early and reliable indicator of apoptosis (Deveraux et al. 1998). A confluent monolayer of cancer (MCF-7, MDA MB-468, Caco-2) cells were incubated in a 6 well plate for 24 hours in DMEM (complete) media. After dosing cells with positive controls (actinomycin-D 4µM, tamoxifen 5µM, anastrazole 5µM) along with pregnane and androstan glycosides according to there specific IC_{50} values for particular cell line, the plates were incubated for further 24 h in incubator at 37°C, 5% CO_2 in air in a humidified atmosphere. Incubations were terminated by rapid aspiration of the cell supernatant followed by washing with cold PBS. The cells were lysed with 1×Laemmli lysis buffer (2.4M glycerol, 0.14M Tris, pH 6.8, 0.21M sodium dodecyl sulphate, 0.3mM bromophenol blue) mixed with β-mecaptoethanol (91:9) and boiled for 5 minutes. The protein lysates were resolved electrophoretically (Bio-Rad electrophoresis apparatus frequency 50/60 Hz with Bio-Rad Mini-Protean Tetra cell reservoir, Singapore) at 200V on 10% denaturing SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-C Extra, GE Health Care, UK). After membrane blocking with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% skimmed dried milk for 1 h at room temperature, the membranes were washed twice with TBS-T and incubated with 10µl PARP rabbit primary antibody (Cell signalling technology, product code 9542, Danvers USA) in 1% Milk in TBS-T (10 ml) for overnight at 4 °C. The membranes were washed three times with TBS-T for 10 min and incubated for 1 h at room temperature with 10µl secondary antibody (Anti-rabbit IgG from Donkey, product code NA 9340, Amersham Biosciences, UK) in 1% Milk in TBS-T (10 ml) for 2h. After extensive washing with TBS-T for 2 h, bands were

detected by ECL reagent. Band intensities were quantified using the ECL system on X-ray film (GE Healthcare, UK).

2.10 DNA fragmentation (ladder) assay

DNA fragmentation assay was conducted using the procedure of Wu et al. (2005) and Gilbert et al., (2007) with some modifications. All three cancer (MCF-7, MDA MB-468, Caco-2) and two normal (HUVEC and U937) cells were cultured at density of $5x10^5$ per well in the presence or absence of pregnane and androstan glycosides according to their estimated IC_{50} for specific cell line for 24 h. Actinomycin-D (4µM), tamoxifen (5µM), Anastrozole (5µM) were used as positive controls. Cells were collected and washed with PBS. The pellet was homogenized in 450µl of lysis buffer (20mM Tris–HCl, pH 8.0, 10mM EDTA, pH 8.0, 0.2% Triton X-100) by repeated pipetting in a microtube and incubated for 10min on ice. The lysates were centrifuged for 15min at 13,000×*g* and to the supernatants, 5µl of 10% SDS solution added. Then samples were incubated at 50 °C for 2h after treating with 5µl RNase A (1mg/ml). Further 5µl proteinase K (2mg/ml) was added and incubated for 2h at 37°C. DNA was precipitated with two volumes of 100% ice cold ethanol and 0.1 volumes of 10M ammonium acetate for 2 h at -70 °C. DNA was pelleted at 12,000×*g* for 15min and washed twice with 70% ice cold ethanol and air-dry for 10 min at room temperature. DNA pellet was dissolved in 30µl of TE loading Buffer at 37 °C, and analyzed by electrophoresis. A 1.5% agarose gel was made by dissolving 1.5 %v/v agarose (cat. 15510-027, Invitrogen, UK) solution in 1×TAE buffer (2M Tris, 1N glacial acetic acid and 0.05M EDTA, pH 8), supplemented with 5µl of GelRed (Biotium Hayward, UK). The solid gel was placed in a tank containing 1× TAE buffer. Isolated DNA was injected into the wells formed after solidification starting with Amplisize molecular marker (50-2,000bp, Cat. 170-8200, Bio-Rad, UK) and 100V current applied for 45 min. After electrophoresis the gel was visualised using the GelDoc system and was photographed through digital camera.

2.10 Materials

Foetal bovine serum (FBS), Penicillin/Streptomycin-L-Glutamine, Dulbeccos Modified Eagles Medium (DMEM), Trypsin-EDTA and Phosphate buffer saline (PBS) were purchased from Fisher (Fisher Scientific Ltd., Leicestershire, UK). All chemical solvents, Silica gel 60 (0.035 – 0.070 mm), actinomycin D (A1410, ~98%) tamoxifen (T5648, ≥99%) and anastrozole (A2736, ≥98%) were purchased from Sigma Aldrich (Sigma Aldrich Company Ltd. Dorset, UK).

2.12 Data presentation and statistical analysis

All data were compiled from a minimum of three experiments. Data for statistical analysis were expressed as the mean ± standard deviation, n (number of experiments). One-way ANOVA with Dunnett's or Tukey's post test, as specified, was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

3. Results

3.1 Activity of the crude extract

A series of eight dilutions of *Caralluma tuberculata* crude extract (10 – 500 μg/ml) were used to determine the concentration-dependent, anti-proliferative effect on three cancer cell lines (MCF-7, MDA-MB-468, Caco-2). The data indicated significant concentration-dependent growth inhibition of the malignant cells after 24 hours with an apparent IC_{50} value of about 100 μg/ml (n=3) with the neutral red uptake (NRU) assay (Figure 1). Similar results were obtained with the MTT assay, where the IC_{50} value was about 200 μg/ml (data not shown).

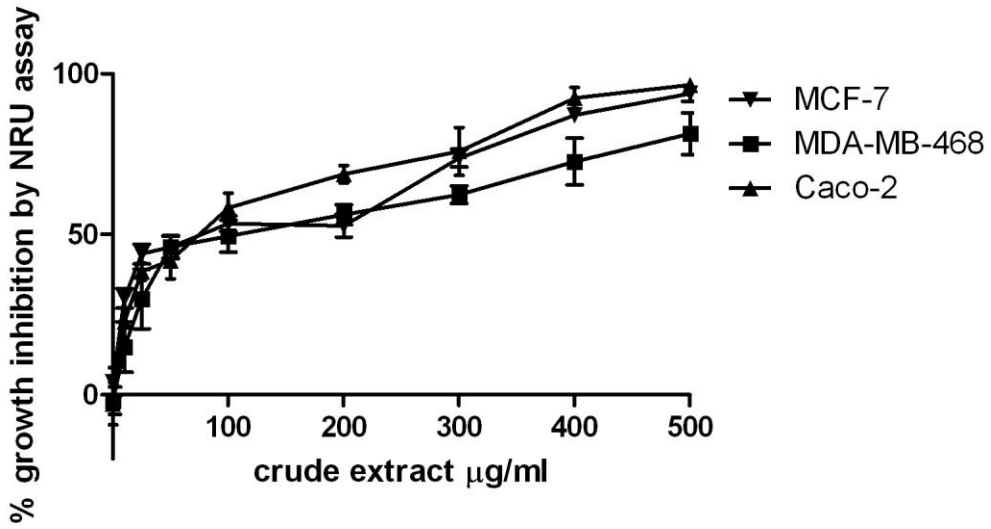


Figure 1. Treatment of cells for 24 hours with the crude extract of Caralluma tuberculata over a range of concentrations. Cell growth was measured using the NRU assay.

3.2 Activity of organic fractions

In a biologically activity-guided fractionation approach, the hexane, $CHCl_3$ EtOAc, MeOH and aqueous fractions of *Caralluma tuberculata* were evaluated at a concentration of 200 μg/ml against MCF-7, MDA-MB-468 and Caco-2 cells. The anti-proliferative activity was significantly concentrated in three fractions ($CHCl_3$, EtOAc and MeOH, n=3 for each fraction, mean ± sd) with similar results obtained

with the NRU assay (Figure 2). The EtOAc fraction had the maximum effect on the cells, reducing growth by 94% ± 4.0 % (n=3) on MCF-7 cells, and 96% ± 7.7% on Caco-2 cells, using the NRU assay. Again, similar results were obtained with the MTT assay: 95% ± 2.6 % inhibition on MCF-7 cells (n=3) and 93% ± 3.9 % inhibition on Caco-2 cells (n=3) (data not shown). MDA-MB-468 cells were less sensitive to each of the fractions, with the ETOAc fraction still the most active, but with only 46% ± 2.8% (n=3) inhibition (NRU assay, Figure 2), and 39% ± 6.4% (n=3) inhibition (MTT assay, data not shown).

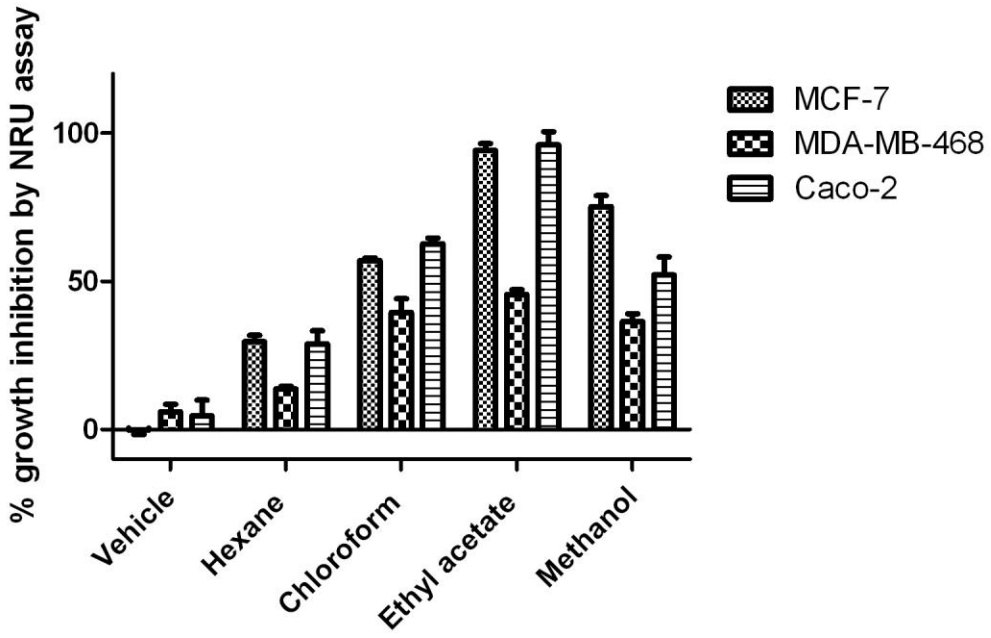


Figure 2. Treatment of cancer cells for 48 hours with specific organic fractions of Caralluma tuberculata at 200 μg/ml. Cell growth was measured using the NRU assay.

3.3 Characterisation of pregnane and androstan glycosides

Compound **1** was isolated as a light-yellowish white, amorphous powder and identified by MS, GC-MS, IR and NMR data as an acylated pregnane glycoside with the molecular formula $C_{48}H_{72}O_{20}$. Therefore, based on the present studies and reported data, the structure of Compound **1** was established as 12-*O*-benzoyl-20-*O*-acetyl-3β,12β,14β,20β-tetrahydroxy-(20S)-pregnan-3-yl *O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-3-methoxy-β-D-ribopyranoside (Fig. 3).

Compound **2** was also isolated as a light greenish-white, amorphous powder and identified as an acylated androstan glycoside with the molecular formula $C_{68}H_{106}O_{26}$, as deduced from ^{1}H, ^{13}C, DEPT,

edited DEPT NMR and ESI-MS (positive). The structure of Compound **2** was established as 7-*O*-acetyl-12-*O*-benzoyl-3β,7β,12β,14β-tetrahydroxy-17β-(3-methylbutyl-*O*-acetyl-1-yl)-androstan-3-yl*O*-β-D-glucopyranosyl-(1→4)-6-deoxy-β-D-allopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymapyranosyl-(1→4)-β-D-cymaropyranoside (Fig.3).

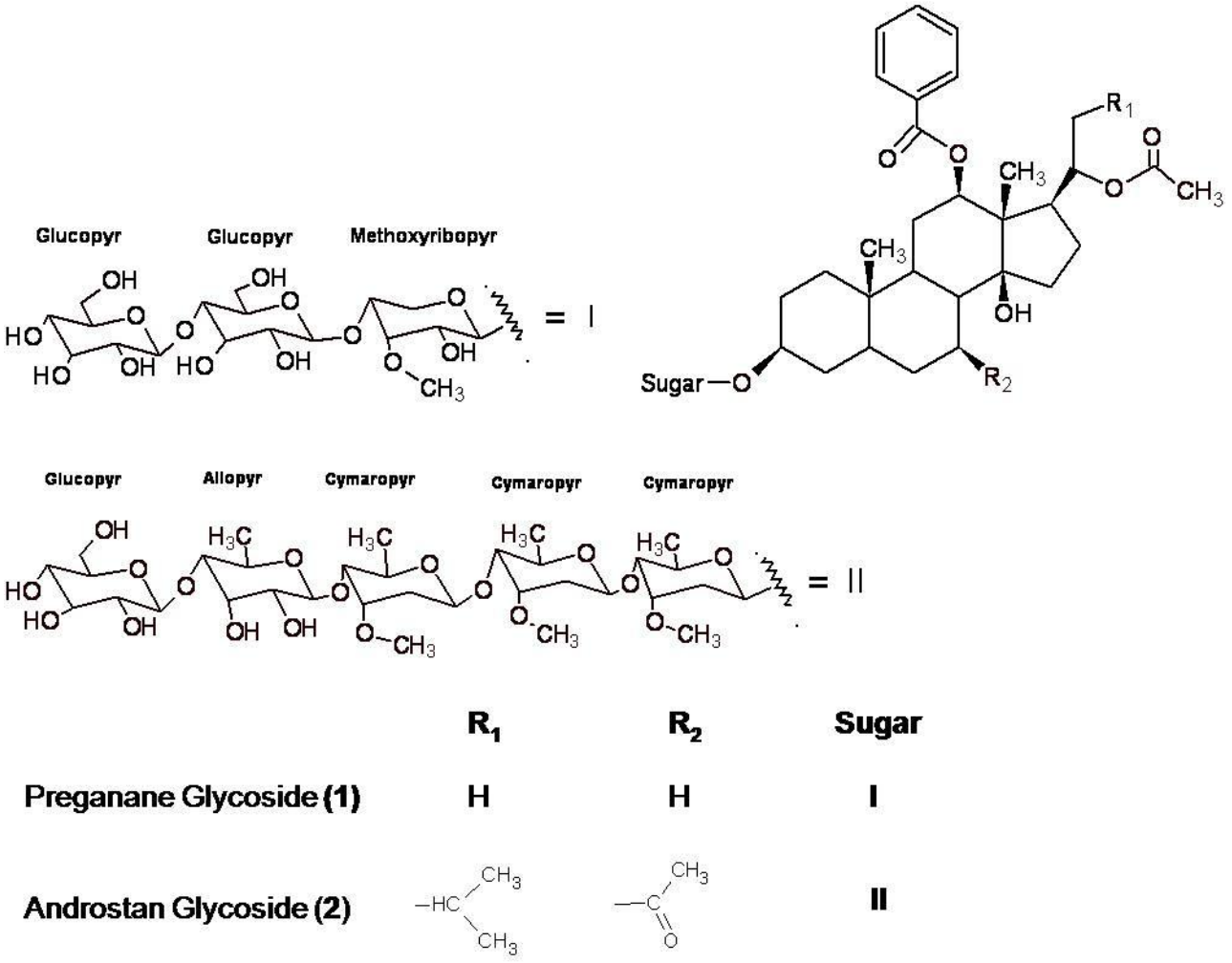


*Figure 3 Structure of pregnane **(1)** and androstan **(2)** glycosides from Caralluma tuberculata*

Compound **1** (pregnane glycoside) and Compound **2** (androstan glycoside) were next tested on MCF-7, MDA-MB-468 and Caco-2 cells. Both compounds were added in the concentration range 0.78 μM – 100 μM for 24 or 48 h incubation, and growth inhibition measured using the MTT and NRU assays. Three positive controls were used: Actinomycin-D (3.98 μM, inhibitor of protein synthesis), tamoxifen (5 μM, estrogen receptor antagonist) and anastrozole (5 μM, aromatase inhibitor).

Compound **1** (pregnane glycoside) inhibited cell growth with the following apparent IC_{50} values, using the NRU assay, after 48 hours: MCF-7 cells 6.25 - 12.5 μM, MDA-MB-468 cells 25 - 50 μM, Caco-2 cells 1.56 – 3.12 μM (Figure 4). Similar data was obtained using the MTT assay, after 48 hours: MCF-7 cells 12.5 - 25.0 μM, MDA-MB-468 cells ~50 μM, Caco-2 cells ~6.25 μM (data not shown). Inhibition of cell growth, relative to vehicle-treated cells, was statistically significant (by one way ANOVA with Dunnett's post-test) at 6.25 μM and higher for pregnane glycoside in MCF-7 and MDA-MB-468 cells, and at all concentrations tested for Caco-2 cells.

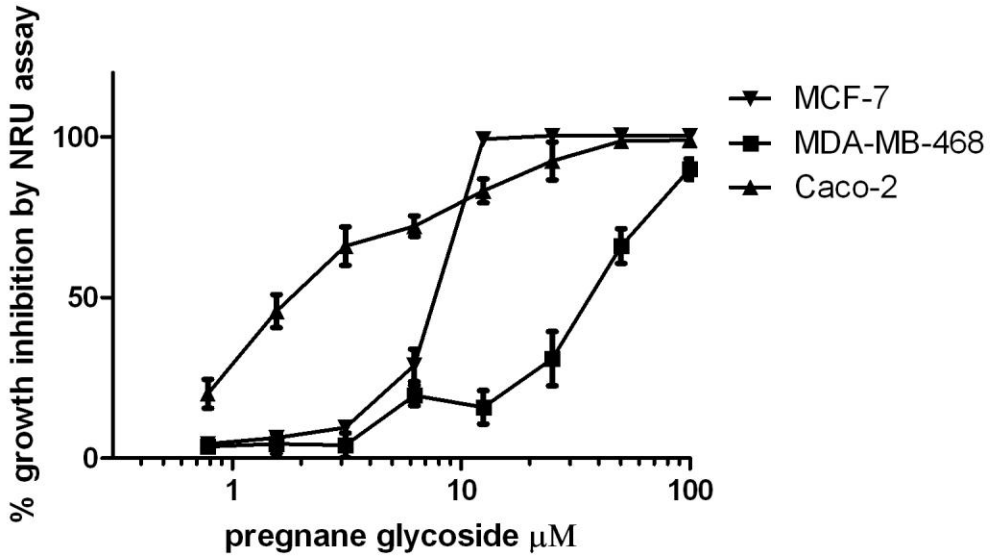


Figure 4. Treatment of cancer cells for 48 hours pregnane glycoside over a concentration range. Cell growth was measured using the NRU assay.

Compound **2** (androstan glycoside) inhibited cell growth with the following apparent IC_{50} values, using the NRU assay, after 48 hours: MCF-7 cells 3.12 - 6.25 μM, MDA-MB-468 cells 25 - 50 μM, Caco-2 cells 3.12 -6.25 μM (Figure 5). Similar data was obtained using the MTT assay, after 48 hours: MCF-7 cells 6.25 - 12.5 μM, MDA-MB-468 cells 25-50 μM, Caco-2 cells 3.12 - 6.25 μM (data not shown). Inhibition of cell growth by androstan glycoside, relative to vehicle-treated cells, was statistically significant at all concentrations in MCF-7 and Caco-2 cells, but from 6.25 μM and higher concentrations for MDA-MB-468 cells.

Growth in the three cell lines was inhibited in response to the positive controls as expected (data not shown). Actinomycin-D inhibited the growth of MCF-7 cells by 82-95% (MTT assay) and 95-100% (NRU assay) after 48 hours. Tamoxifen completed inhibited the growth of MCF-7 after 48 hours, using

either MTT or NRU assay, but was less effective, as expected, on the growth of MDA-MB-468 cells (45% MTT assay, 63% NRU assay). Anastrozole was only moderately inhibitory on MCF-7 cells (46% MTT assay, 36% NRU assay) but much more effective on MDA-MB-468 cells (87% MTT assay, 93% MRU assay).

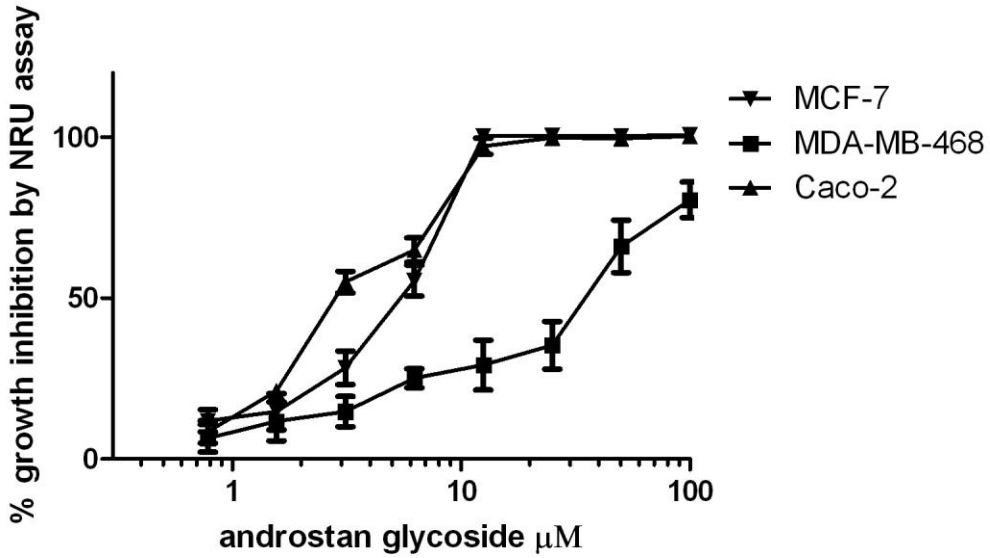


Figure 5. Treatment of cancer cells for 48 hours androstan glycoside over a concentration range. Cell growth was measured using the NRU assay.

Further experiments were performed to investigate the mode of action of the pregnane glycosides on cells, for example, did compounds 1 and 2 stimulate apoptosis or necrosis, and was the activation of caspases involved? The three cell lines (MCF-7, MDA-MB-468, and Caco-2) received pregnane glycosides at their IC_{50} concentrations for 12 hours in experiments designed to show either a) DAPI nuclear staining, b) caspase involvement as judged by the effect of the pan-caspase inhibitor Z-VAD-FMK, c) induction of apoptosis as shown by cleavage of poly ADP-ribose polymerase (an important enzyme in DNA repair), or d) induction of apoptosis and nuclear fragmentation as shown by a DNA ladder assay.

In the first experiment, cells received either glycoside at the IC_{50} concentration for 12 hours: pregnane glycoside MCF-7 20 μM, MDA-MB-468 50 μM, Caco-2 10 μM; androstan glycoside MCF7 12.5 μM, MDA-MB-468 50 μM, Caco-2 6.25 μM. Cells were then stained with the blue nuclear dye DAPI and visualised on a confocal microscope (see Methods). In the case of the MCF-7 and Caco-2 cells, addition of the pregnane or androstan glycoside resulted in shrinkage of cells, a reduction in cell number and evidence of nuclear fragmentation, all characteristics of apoptosis, when compared to untreated cells or

cells treated with vehicle (0.01% DMSO) (Figure 6). MDA-MB-468 cells, however, appeared fewer in number, after treatment with the steroidal glycosides, but those remaining cells appeared a little larger than untreated or vehicle-treated cells, suggesting swelling characteristic of necrosis.

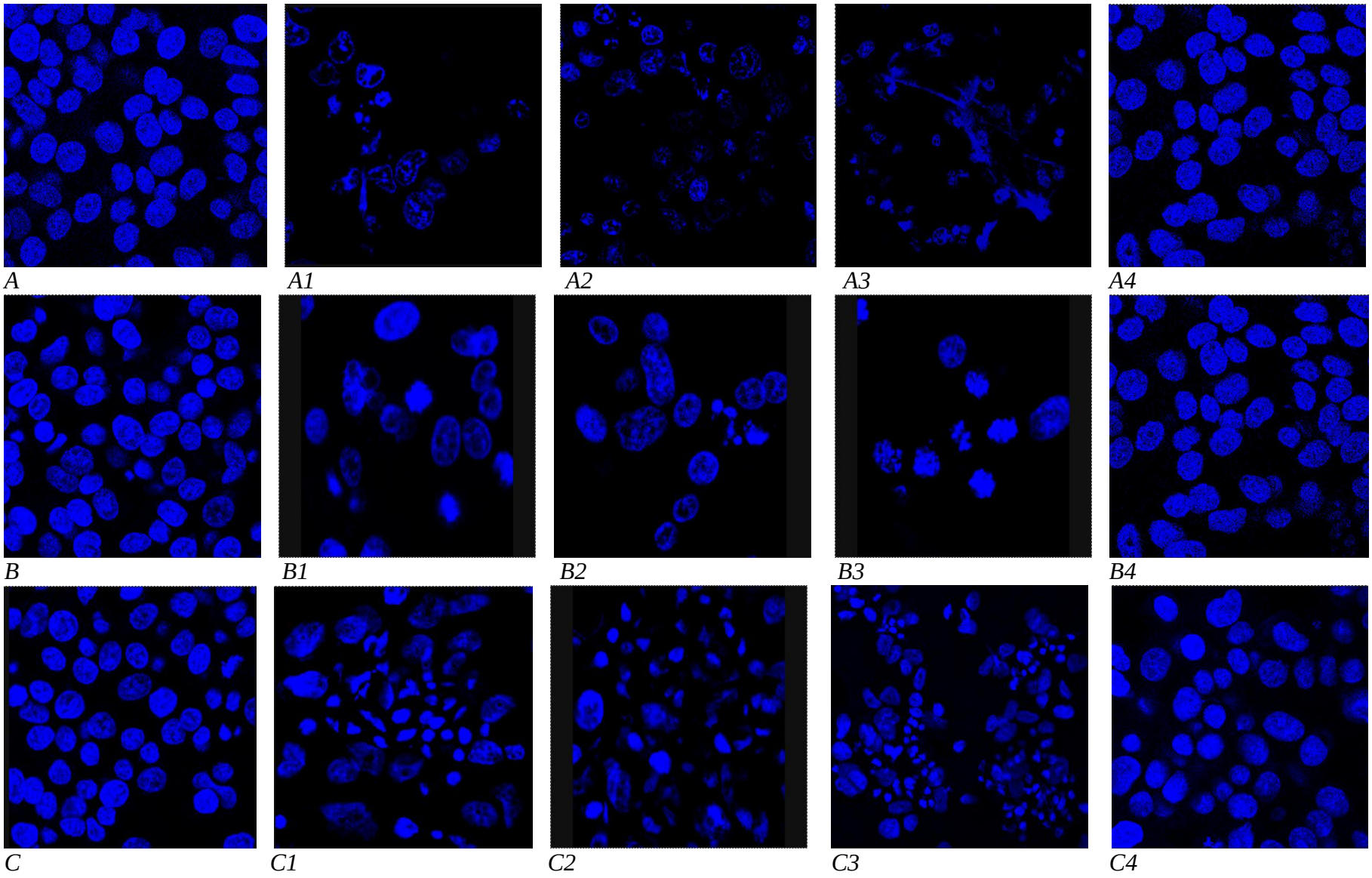


Figure 6. Treatment of cancer cells for 24 hours with vehicle (0.01% DMSO) and pregnane or androstan glycosides at the IC_{50} concentration for the specific cell type with positive control as Actionmycin –D. Cell nuclei were stained with DAPI where:

MCF-7 Cells: A- Control, A1-Pregnane (20μM), A2- Androstan (12.5μM), A3- Actinomycin-D (4μM), A4- Vehicle Control.

MDA MB-468 Cells: B- Control, B1-Pregnane (50μM), B2- Androstan (50μM), B3- Actinomycin-D (4μM), B4- Vehicle Control

Caco-2 Cells: C- Control, C1-Pregnane (10μM), C2- Androstan (6.25μM), C3- Actinomycin-D (4μM), C4- Vehicle Control

In the second experiment, the involvement of caspases was assessed by use of the pan-caspase inhibitor Z-VAD-FMK (50μM). Cells were treated as follows for 24 hours with pregnane glycoside at its IC_{50} value depending on cell type: MCF-7 20μM, MDA 50μM, Caco-2 10μM, and similarly for androstan glycoside: MCF-7 12.5μM, MDA-MB-468 50μM, Caco-2 6.25μM. In all cases the inhibition in growth by the novel steroidal glycosides was reduced by pre-treatment with Z-VAD-FMK, as was the effect of actinomycin-D (Figure 7, MCF-7 cells shown, very similar results were obtained for MDA-MB-468 and Caco-2 cells, data not shown). Vehicle had no effect on cell growth, but Z-VAD-FMK had a slight stimulatory effect, perhaps as a consequence of inhibiting a sub-population of untreated cells in apoptosis. All treatments, including those with Z-VAD-FMK, were statistically significant to vehicle-treated controls (one way ANOVA with Tukey's post-test).

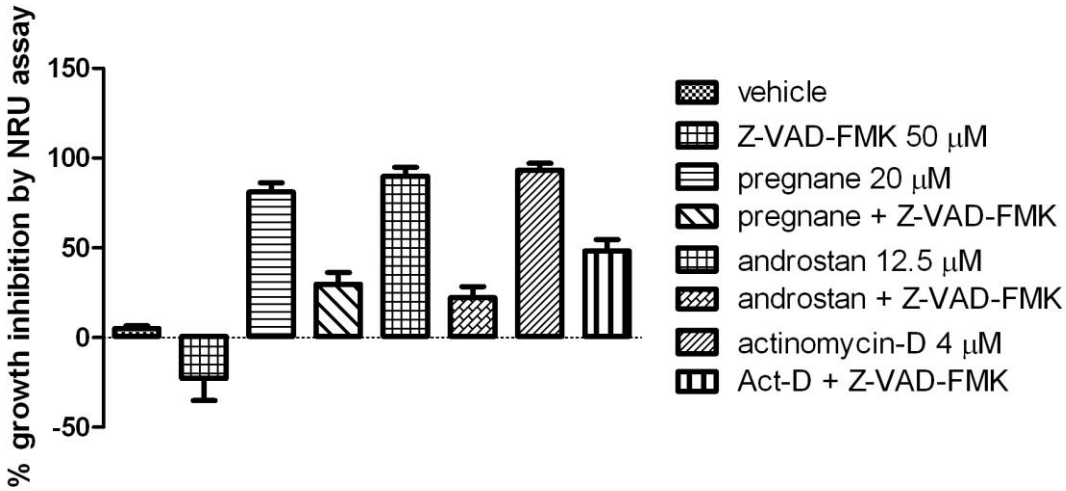
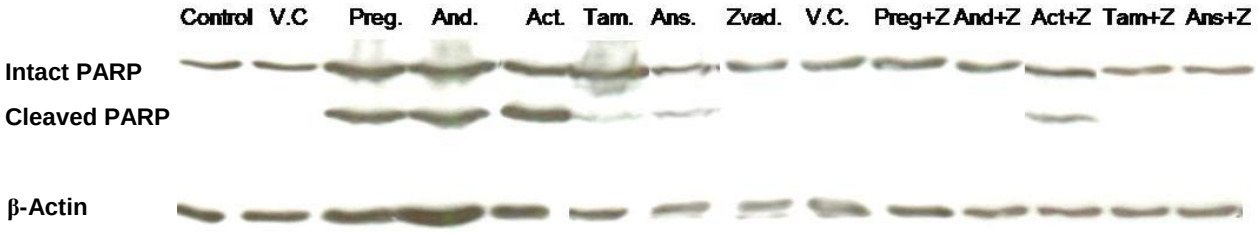


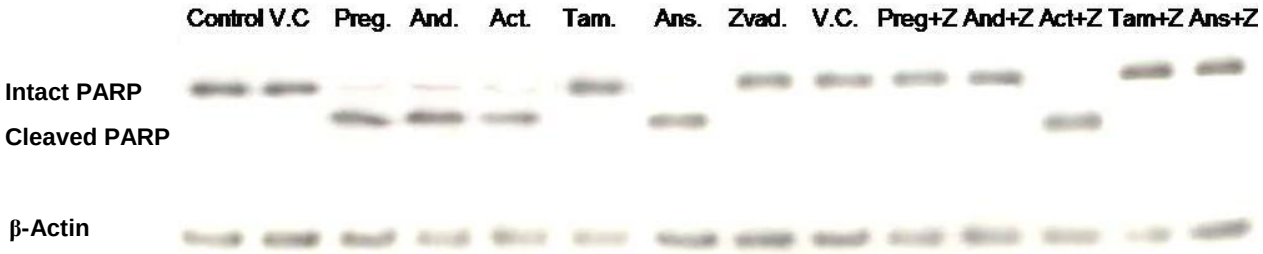
Figure 7. Treatment of cells for 48 hours with pregnane glycoside (compound 1) or androstan glycoside (compound 2) with or without the pancaspase inhibitor Z-VAD-FMK. Cell growth was measured using the NRU assay.

In the third experiment, we used Western blots to detect cleaved PARP (see Methods) as a reliable marker of apoptosis. Cells were treated for 24 hours as follows: pregnane glycoside MCF-7 20μM, MDA-MB-468 50 μM, Caco-2 10μM; androstan glycoside MCF7 12.5μM, MDA-MB-468 50μM, Caco-2 6.25μM. The pregnane and androstan glycosides cleaved PARP, as did the positive controls actinomycin D, anastrozole, and tamoxifen (Figure 8). PARP in untreated or vehicle-treated cells was unaffected. Z-VAD-FMK reduced PARP cleavage to the pregnane or androstan glycosides in all three cell lines, and the positive controls, although Actinomycin-D was more resistant to its effects.

MCF-7 Cells



MDA MB-468 Cells



Caco-2 Cells

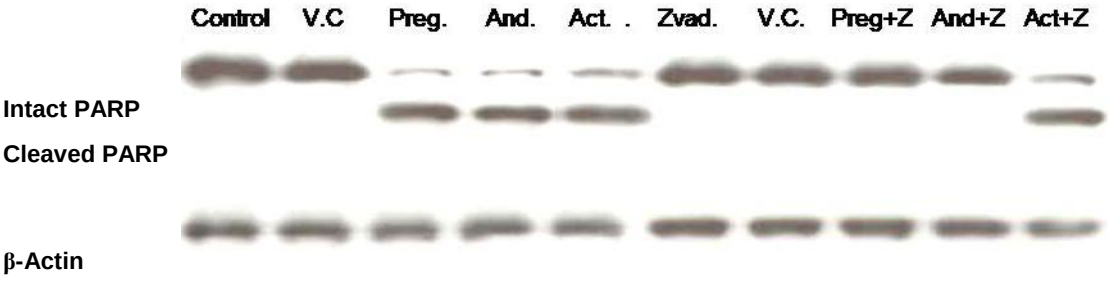


Figure 8. Detection of PARP (intact and cleavage) and β-actin, in lysates of cells treated for 24 hours with vehicle (V.C.) pregnane glycoside (compound 1), androstan glycoside (compound 2), Actinomycin-D (Act.), tamoxifen (Tam.), Anastrozole (Ans.), Z-VAD-FMK, and combinations thereof.

In a fourth experiment, cells were treated with the IC_{50} concentrations of pregnane and androstan glycosides as before, and a DNAase ladder assay was performed. Caspase-dependent PARP cleavage in

response to pregnane or androstan glycosides was confirmed with the use of Z-VAD-FMK**.** Furthermore, neither steroidal glycoside had an effect on DNA fragmentation in HUVEC or U937 cells (Fig. 9).

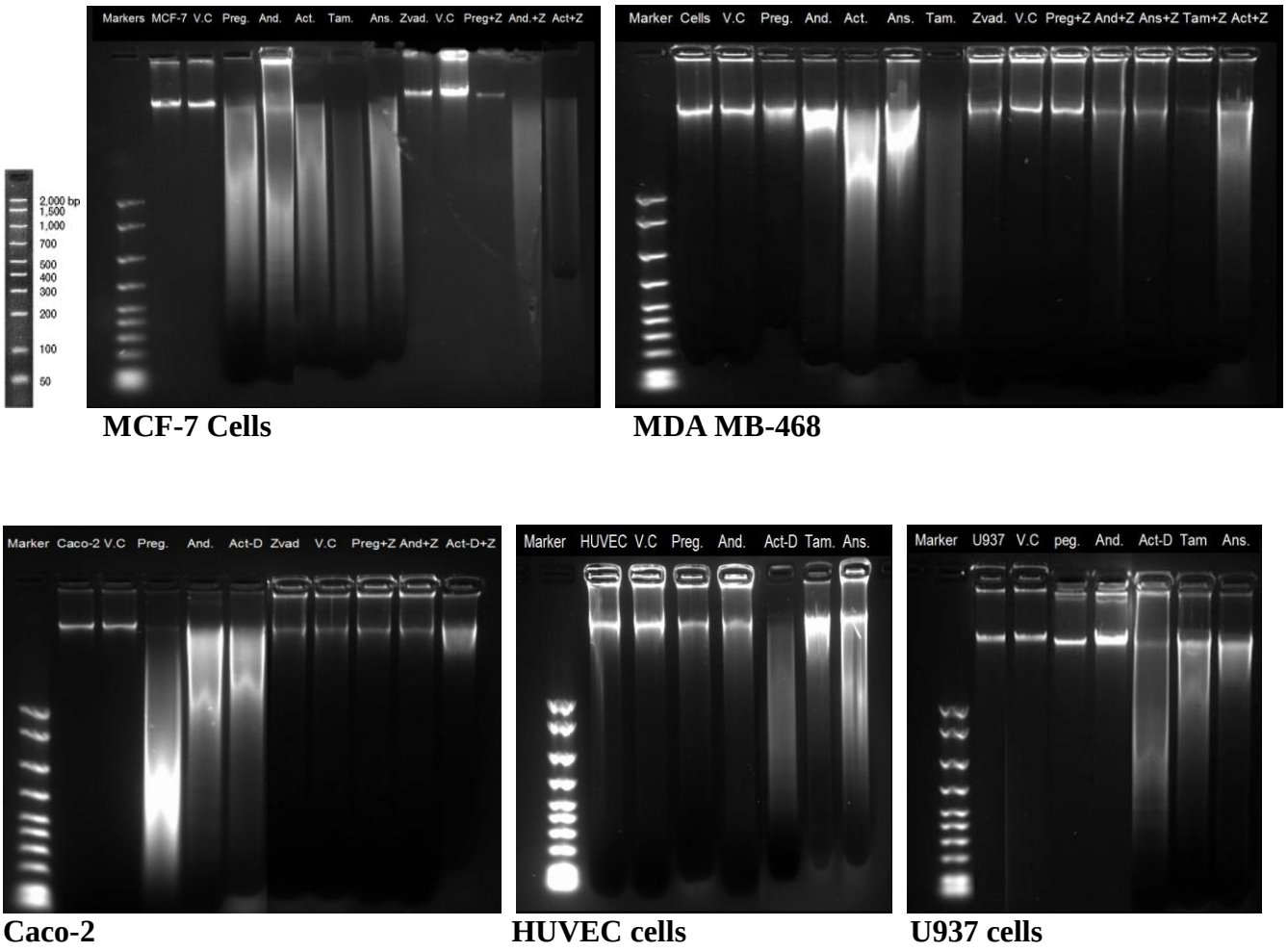


Figure 9. Detection of nuclear fragmentation by a DNA ladder assay in cells after 48 hours treated with vehicle (V.C.) pregnane glycoside (compound 1), androstan glycoside (compound 2), actinomycin D (Act.), tampoxifen (Tam.), Anastrozole (Ans.), Z-VAD-FMK, and combinations thereof.

DISCUSSION

We have identified two new steroidal glycosides that have cytotoxic effects on an estrogen-dependent breast cancer cell line, a colon cancer cell line, and a slightly lesser effect on an estrogen-independent breast cancer cell line. The results of two commonly used cytotoxicity assays (NRU and MTT) confirmed closely with one another and showed that Caco-2 cells were the most sensitive to the pregnane and androstan glycosides, with apparent IC_{50} values in the range 1.56 – 6.25 μM. MCF-7 cells were the next sensitive with IC_{50} values in the range 3.12 - 25.0 μM. The MDA MB-468 cells were the least sensitive with IC_{50} values in the range 25.0 – 50.0 μM. Thus, compounds **1** and **2** have apparent IC_{50} values across three cell lines of <25 μM, or in μg/ml of 24.22 μg/ml for pregnane glycoside (compound **1**) and 33.47μg/ml for androstan glycoside (compound **2**). Other pregnane glycosides isolated from *Caralluma* species (Abdel-Sattar *et al* 2009) had IC_{50} values of ~20 μg/ml on the growth of MRC5 cells. Most remarkably, twenty seven pregnane glycosides isolated from *Caralluma dalzielii* had sub-micromolar IC_{50} values when tested on J774.A1 murine monocyte/macrophage, WEHI-164 murine fibrosarcoma, and HEK-293, human epithelial kidney cells, after 72 hours using the MTT assay (De Leo *et al* 2005). At present we do not have an explanation for this great difference in potency between pregnane glcyosides from that study and our own.

We have begun to characterise the activity and the possible mechanisms of action of the pregnane and androstan glycosides isolated in this study. All of the measures of cytotoxic activity (MTT, NRU, DAPI, PARP cleavage, DNA ladder) on MCF-7, MDA MB-468 and Caco-2 cells were inhibited by pre-treatment with the pan-caspase inhibitor Z-VAD-FMK. Our experiments therefore clearly showed that the pregnane and the androstan glycosides induced caspase-dependent apoptosis in MCF-7 and Caco-2 cells, while the MDA MB-468 cells were less sensitive. Interestingly, HUVEC and U937 cells were less affected by the pregnane and andostan glycosides, at least with regard to DNA fragmentation, the assay used to assess their effect. Possibly, the glycosides may show some selectively between malignant and normal cells, but further work is necessary. It is also presumed that the glycoside is cleaved by cell surface enzymes, such as lactase phorizin hydrolase, to allow the steroid to cross the plasma membrane and enter the cell.

Caspases are calcium-dependent enzymes activated *via* two basic pathways, extrinsic (e.g. ligation of so-called cell surface death receptors), or intrinsic (e.g. damage to mitochrondria), with caspase 3 particularly important in triggering cleavage of PARP and DNA fragmentation, both of which were end points detected in our experiments. Once PARP is cleaved, the cell is irreversibly committed to

apoptosis. Pregnane glycosides are structurally similar to cardiac glycosides such as digoxin (Deepak et al 1997), which inhibit calcium exchangers, thereby leading to a prolonged increase in intracellular calcium concentration and stimulation of caspases and apoptosis. Another possible mechanism of action is the activation of the steroid and xenobiotic receptor (SXR), an orphan steroid receptor, which induces apoptosis in breast cancer cells (Verma *et al* 2009). Further studies are required, but the activation of the SXR is an exciting possibility.

The steroidal glycosides isolated in this study are novel but have some similarity to compound 46 (Bouceroside BDO, C_{51} H_{76} O_{16}), and compound 47 (Bouceroside BNC, C_{51} H_{78} O_{16}) isolated from *Boucerosia aucheriana* and *Caralluma tuberculata* which is part of the *Asclepiadaceae* family (Deepak *et al* 1991), but no activity on tumour cells has been reported, so a comparison with our data is not possible. Importantly, the pregnane glycoside (compound **1**) we report here shares the structural skeleton of a synthetic aromatase inhibitor (compound **4a**, Cepa *et al* 2008) and thus suggests aromatase inhibition as another possible mode of action of compound **1** to be investigated.

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Supplementary Data

Compound 1 and 2, IR, MS, NMR and acid hydrolysis data etc.

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