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Evaluation of anti-tumour properties of two depsidones – Unguinol and Aspergillusidone D – in triple-negative MDA-MB-231 breast tumour cells

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ABSTRACT

There is an ongoing search for new compounds to lower the mortality and recurrence of breast cancer, especially triple-negative breast cancer. Naturally occurring depsidones, extracted from the fungus *Aspergillus*, are known for their wide range of biological activities such as cytotoxicity, aromatase inhibition, radical scavenging, and antioxidant properties. Research showed the potential of depsidones as a treatment option for hormone receptor-positive breast cancer treatment, yet its effects on hormone receptor-negative breast cancer are still unkown.

This study, therefore, investigated the potential of two depsidones (Unguinol and Aspergillusidone D) to induce apoptosis, cell cycle arrest and cytotoxicity, and reduce cell proliferation in the triple-negative MDA-MB-231 breast cancer cell line. Results were compared with the effects of the cytostatic drug doxorubicin, antimitotic agent colchicine and endogenous hormones 17β-estradiol, testosterone and dihydrotestosterone.

The cytostatic drugs and hormones affected the MDA-MB-231 cell line comparable to other studies, showing the usefulness of this model to study the effects of depsidones on a triple-negative breast cancer cell line. At sub μM levels, Unguinol and Aspergillusidone D did not influence cell proliferation, while cell viability was reduced at concentrations higher than 50 μM. Both depsidones induced apoptosis, albeit not statistically significantly. In addition, Unguinol induced cell cycle arrest in MDA-MB-231 cells at 100 μM.

Our research shows the potential of two depsidones to reduce triple-negative breast cancer cell survival. Therefore, this group of compounds may be promising in the search for new cancer treatments, especially when looking at similar depsidones.

1. Introduction

Even though breast cancer has a high priority in cancer research, it remains one of the leading causes of cancer deaths in women worldwide [1]. 10–20% of all breast cancers are hormone receptor (triple) negative. This type of cancers is more likely to affect carriers of the BRCA1 gene mutation, women before the age of 40–50 and/or African American and Hispanic women [2,3]. Even though triple-negative breast cancer is biologically highly heterogeneous [4], all subtypes do not express the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 receptor (HER2) per definition. As a result, cancer growth is not influenced by the hormones estradiol and progesterone, the HER2 protein or by adjuvant hormonal treatment (such as estrogen receptor antagonists and aromatase inhibitors). As a result, the most common treatment of triple-negative breast tumours is the use of chemotherapeutic drugs like paclitaxel, doxorubicin or cyclophosphamide as (neo)adjuvant systemic treatment, which can be followed by radiation therapy [5]. However, serious long-term side effects like cardiac failure have been linked to the use of these cytotoxic drugs [6,7]. Due to the limited treatment options as well as the aggressive nature of both the cytostatics and triple-negative breast tumours, the 5-year survival for triple-negative breast cancer patients is significantly lower compared to hormone receptor-positive breast cancers [8]. For hormone receptor-negative breast cancer tumours, the general consensus is that these tumours need to be treated *via* a multi-target approach to reduce mortality and morbidity, remove residual

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cancer cells and prevent recurrence [9].

During the last decades, the anti-tumour properties of naturally occurring compounds have been of increasing interest. Among these are natural compounds that have been isolated from *Aspergillus unguis CRI282-03*, a fungus found in marine organisms and their environment. From this fungus, several classes of compounds, like protubonines, cerebroside analogues and depsidones have been extracted [10–12].

Depsidones are organic compounds comprising of depside-like esters and cyclic ethers. These naturally occurring depsidones are known to have a variety of biological activities that could be therapeutically relevant [13] including anti-tumour activity in the triple-negative breast cancer cell line MDA-MB-231 [10,11]. In addition, antimalarial activity against *Plasmodium falciparum,* cytotoxicity of human breast cancer (BC1) cells [14], antibacterial activity against the multidrug-resistant *Staphylococcus aureus* [15], cytotoxicity of MDA-MB-231 cells, inhibition of aromatase, radical scavenging and antioxidant activities (the latter three tested without cell systems) [16], antiviral (HIV-1-IN) and antifungal (*Aspergillus terreus* and *Fusarium osysporum*) activity [17–19] have been reported. Apoptotic properties of depsidones have also been described in rat thymocytes [20]. Recently, Chottanapund, et al. identified two depsidones, Unguinol and Aspergillusidone A, that reduce cell growth and inhibit aromatase activity in the hormone receptor-positive breast cancer cell line T47D co-cultured with primary human breast fibroblasts [21].

Given the potential of depsidones with regards to hormone receptor-positive breast cancer, the current research investigated the potential anti-cancer properties of depsidones for triple-negative breast cancer cells. In short, this study investigated whether Unguinol and the novel depsidone Aspergillusidone D (Fig. 1) affected cell proliferation, cell viability, cell cycle arrest and apoptosis in the triple-negative breast cancer cell line MDA-MB-231. The potency of the depsidones were compared with effects of doxorubicin, the conventional cytostatic treatment against triple-negative breast cancer, and colchicine, a known mitotic inhibitor. In addition, the effects of depsidones were assessed against the effects of endogenous hormones surrounding breast (cancer) cells, *i.e.* 17β-estradiol, testosterone and dihydrotestosterone.

2. Materials and methods

2.1. Chemicals

The depsidones Unguinol and Aspergillusidone D were provided by the Chulabhorn Research Institute and have been biosynthesized according to Sureram et al. [12]. The other compounds tested, *i.e.* 17β-estradiol (purity > 98%), testosterone C-III (purity > 98%), 5α-androstan-17β-ol-one C-III or dihydrotestosterone (purity 97.5%), colchicine (purity ± 95%) and doxorubicin (purity > 98%) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). These compounds were dissolved and diluted in dimethyl sulphoxide (DMSO) Hybri-max from Sigma-Aldrich (St. Louis, MO, USA) and added to the exposure medium at < 0.5% (v/v).

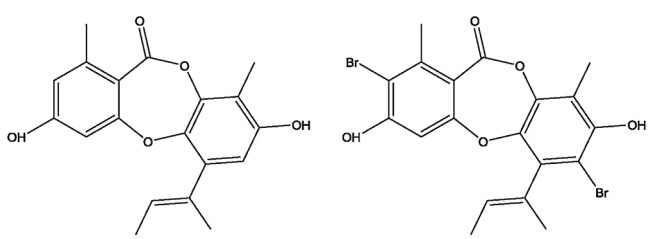

Fig. 1. Chemical structures of Uguinol and Aspergillusidone D.

2.2. Breast cancer cell culture

The human triple-negative breast cancer cell line MDA-MB-231 was obtained from ATCC (Rockville, MD, USA). This epithelial breast cancer cell line was established from a female with metastatic mammary adenocarcinoma. The cell line is highly aggressive, invasive and poorly differentiated as it poorly expresses the estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2) [22]. In addition, the cell line has limited tight junctions and Ki-67 expression. Moreover, these cells have altered glycoproteins involved in cell-cell interactions, while expressing markers related to epithelial-mesenchymal transition [23]. The MDA-MB-231 cell line is now classified to belong to the mesenchymal stem-like subtype [4]. While triple-negative breast cancer is highly heterogeneous and all 7 subtypes differ in biology and response to treatment [24], the invasive MBA-MB-231 cell line is most commonly used cell line for studying triple-negative breast cancer [25].

All cell culture supplies were obtained from Gibco, Thermo Fisher Scientific, Waltham, MA, USA. Cells were grown under sterile conditions in culture medium comprising of Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine at seeding concentration of 5×10^4 cells/mL. The cells were kept at 37 °C in a humidified atmosphere with 5% CO_2, unless otherwise stated, in 25 or 75 cm^2 culture flasks (Thermo Fisher Scientific, Waltham, MA, USA). The cells were passaged when they reached 80% confluence, which was typically after 2–3 days.

At the start of the experiments, cells were plated at a density needed corresponding to the different experimental endpoints, as specified below. While doing this, the medium was changed into plating medium: DMEM- low glucose medium (Sigma-Aldrich, St. Louis, MO, USA) without phenol red containing 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine. Cells were kept overnight to adhere to the plate. After 24 h, the plating medium was removed, and the assay medium was added. Assay medium consisted of DMEM- low glucose, 10% dextran/charcoal-stripped serum, 1% penicillin/streptomycin and 1% L-glutamine, spiked with the test compounds. The cells were exposed to the test compounds for 1, 3 or 5 days, depending on the type of experiment.

2.3. Cell proliferation

To test if the compounds could reduce cell proliferation, 100 µL of MDA-MB-231 cells were plated in a 96-well plate at a low density of 20,000 cells/mL. A low density was used to make sure no overcrowding of the cells would take place during prolonged exposure. After 24 h, medium was removed and cells were exposed to low concentrations of the test compounds (0.01–5 µM Unguinol or Aspergillusidone D, 5–20 pM 17β-estradiol, 5–20 µM testosterone or dihydrotestosterone, or 0.1% DMSO (vehicle control)) for 5 days. Concentrations were based on earlier results from Chottanapund et al. [21,26]. During the 5 days of exposure, cell viability was visually determined and it was established that cell growth was not influenced by medium supplement depletion due to bolus exposure. After 5 days of exposure, MTT (5 mg MTT/mL in PBS) was added to each well for 4 h (10 times diluted in well medium) [21,26]. Next, the medium was aspirated and DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 560/670 nm using the 96-wells microplate reader (Spectramax plus 384, Molecular Devices, California, USA).

2.4. Cell viability

To determine the effects of 17β-estradiol, testosterone, dihydrotestosterone, Unguinol and Aspergillusidone D on cell viability, MDA-MB-231 cells were exposed for 3 days after which an MTT assay was performed [21]. As a positive control for cell viability, cells were

exposed to doxorubicine. In short, 100 µL of MDA-MB-231 cells were plated in a 96-well plate at a concentration of 60,000 cells/mL. After 24 h, the medium was removed and cells were exposed for 72 h to assay medium containing 0–100 µM 17β-estradiol, 0–500 µM testosterone or dihydrotestosterone, 0–10 µM doxorubicin or 0.1% DMSO (vehicle control). Unguinol and Aspergillusidone D were tested at final concentrations of 0–200 µM for 3 days before an MTT assay was performed as stated above. For all compounds, preliminary concentration range-finding experiments were performed to determine the concentrations needed to induce cytotoxicity.

2.5. Cell cycle arrest

Cell cycle arrest was studied in MDA-MB-231 cells that were plated in 6-well culture plates with 2.5 mL of 400,000 cells/mL per well. After 24 h, the medium was removed and cells were exposed to assay medium containing 50 µM 17β-estradiol, 50 µM testosterone, 50 µM dihydrotestosterone or 0.05% (v/v) DMSO (vehicle control). As a positive control for cell cycle arrest, cells were exposed to 3 µM colchicine. Unguinol and Aspergillusidone D were tested at concentrations of 100 and 60 µM, respectively. The depsidone concentrations were chosen based on cell viability assays done in this study.

After 24 h of exposure, assay medium and the cells were harvested and centrifuged (5 min, 2000 rpm, 4 °C), after which the supernatant was removed. The cells were re-suspended in 1000 µL PBS and transferred to an Eppendorf tube before centrifugation (5 min, 5000 rpm, 4 °C). Again, the supernatant was removed and 300 µL of PBS was used to re-suspend the cells before adding 700 µL of 70% ice-cold ethanol. After mixing, this suspension was incubated for 40 min at 4 °C to fixate the cells. This was again followed by centrifugation (5 min, 5000 rpm, 4 °C), removal of the supernatant, re-suspension in 1000 µL PBS and centrifugation (5 min, 5000 rpm, 4 °C). After centrifugation, the supernatant was removed and 500 µL propidium iodine solution (PBS with a final concentration of 0.05 mg/mL propidium iodine + final concentration 5.0×10^{-4} mg/mL RNAse stock solution (DNAse free)) was added to each sample. Samples were incubated for 15 min at room temperature and transferred to FACS tubes before cell cycle phase distributions were measured on the DB FACSCanto ™ flow cytometer (BD Biosciences, San Diego, CA, USA). 30,000 cells were analysed per sample at a middle flow rate, as described previously [27]. Duplicates and triplicates were tested in 3 or 4 independent experiments. Cells were sorted in the cell cycle phases gated as G0/G1, S and G2/M. Data was analysed using ModFit LT™software.

2.6. Apoptosis

To determine the percentage of apoptotic cell populations induced after exposure to the test compounds, the cells were treated following the described method for the cell cycle arrest assay until cells were harvested. As a positive control for apoptosis, cells were exposed to 0.5 µM doxorubicin. Next, apoptosis was determined using the Muse™ Annexin V & Dead Cell Assay kit (Merck MCH100105) according to the manufacturer's description. In short, after 24 h of exposure, the cells were harvested and centrifuged (5 min, 2000 rpm, 4 °C) after which the supernatant was removed. The cells were re-suspended in 1000 µL DMEM with 10% FBS. 100 µL of this suspension was transferred to an Eppendorf tube containing 100 µL of Muse™ Annexin V & Dead Cell Reagent (7-aminoactinomycin-D (7-AAD) and Annexin V dyes) followed by incubation for 20 min at room temperature in the dark. After this incubation period, the amount of phosphatidylserine (PS) molecules translocated to the outer membrane and the membrane structural integrity were measured on a Muse® Cell Analyser (Merck Millipore, Millipore Sigma, Merck, Darmstadt, Germany).

2.7. Data analysis

Data on cell viability and cell proliferation were corrected for background before control wells were checked for outliers (± 2*SD). Thereafter, exposed wells were normalized to the average control value of the corresponding plate, correcting for unintendedly induced artefacts. Conditions were averaged per plate (technical replicate per plate) before the average of multiple experiments was taken.

To evaluate apoptosis and cell cycle arrest, averages were taken from the technical replicates for the different stages of apoptosis and different arrest phases. Thereafter, the average of multiple experiments was taken. 1 of the 4 technical apoptosis replicates of Aspergillusidone D (N = 2) was excluded from the analysis. In the cell cycle arrest data, 1 DMSO and 1 colchicine experimental replicate were considered to be outliers (± 2*SD).

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). Concentration-response curves were calculated using the GraphPad Prism nonlinear regression sigmoidal dose-response curve fitting module. All data in the figures are expressed as mean ± SEM for n technical replicates per plate of N plates. Biological variation is shown as 1xSD (10% for cell proliferation and cell viability assays). To test statistical significance, one-way and two-way ANOVA followed by Bonferroni's multiple comparisons tests were used with $p < 0.05$ (based on experimental replicates (N)). Cell viability curves were fitted using non-linear regressions, with a bottom = 0 constraint for all compounds that did not reach 0% viability, using GraphPad Prism 7.0. IC_{50} values are depicted with 95% confidence intervals [CI].

3. Results

3.1. Effect of hormones and depsidones on the proliferation of the MDA-MB-231 cell line

To determine whether the cell proliferation of the MDA-MB-231 cell line was influenced by the steroid hormones 17β-estradiol, testosterone and dihydrotestosterone, and depsidones Unguinol and Aspergillusidone D, cells were exposed for 5 days. As can be seen in Fig. 2, both the hormones and the depsidones did not induce relevant effects (*i.e.* exceeding the variation of vehicle control and statistically significantly different from vehicle control) on the proliferation of the hormone receptor-negative breast tumour cells. As no significant effects on cell proliferation were seen, we did not differentiate between anti-proliferative or cytotoxic effects in subsequent experiments.

3.2. Effect of doxorubicin, hormones and depsidones on the viability of MDA-MB-231 cells

All compounds induced cytotoxicity in MDA-MB-231 cells, yet at divergent concentrations (Fig. 3). Doxorubicin most potently inhibits cell viability with an IC_{50} value of 0.025 µM [0.016–0.034]. 17β-Estradiol inhibits cell viability with comparable potency to the depsidone Aspergillusidone D (IC_{50} values of 42 µM [39–44] and 49 µM [45–54], respectively). In addition, the effect concentration for the inhibition of cell viability for Unguinol is with 81 µM [74–89] significantly higher compared to that of Aspergillusidone D. The androgens testosterone and dihydrotestosterone showed the lowest inhibition of cell viability (IC_{50} values of 202 µM [166–236] and 106 µM [96–116], respectively).

3.3. Effect of colchicine, hormones and depsidones on MDA-MB-231 cell cycle arrest

To determine whether the reduction in cell viability was due to an arrest in the cell cycle, cell cycle status was determined after a 24-h exposure to the hormones or depsidones. 17β-Estradiol and the positive control for cell cycle arrest in the G2/M phase, colchicine, induced a

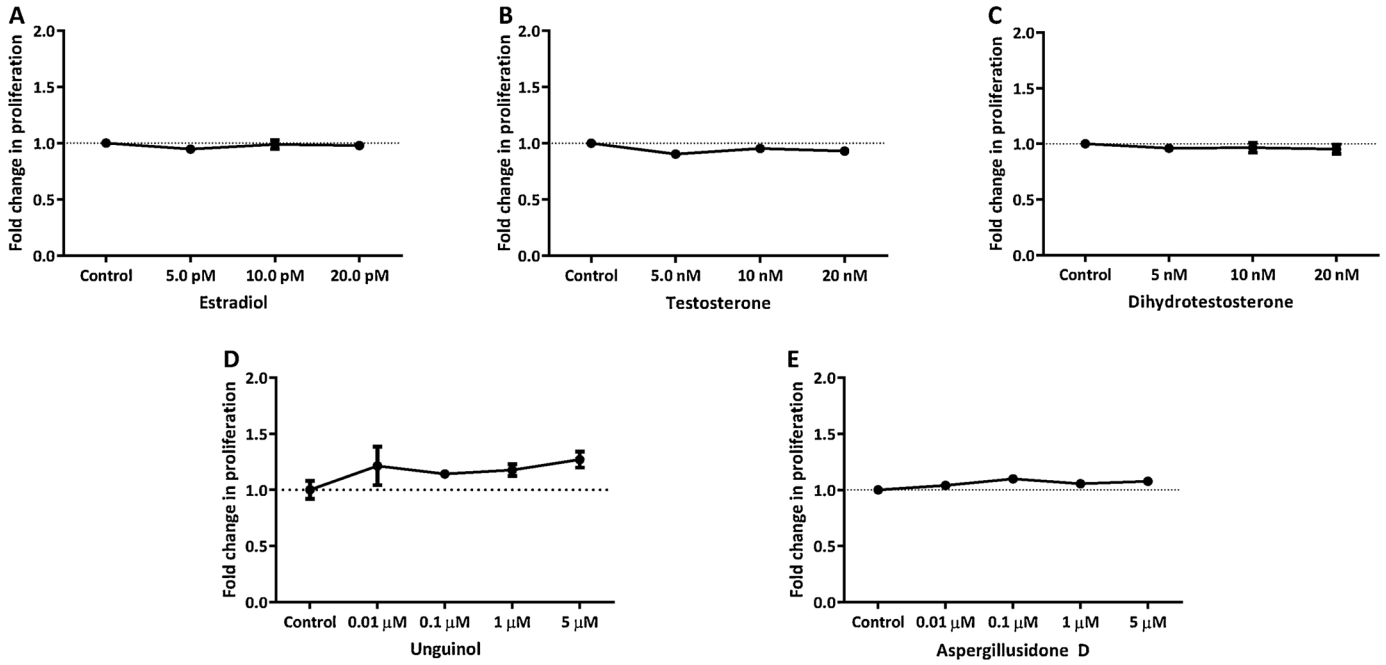

Fig. 2. Cell proliferation of MDA-MB-231 triple-negative breast tumour cells exposed for five days to (A) 17β-estradiol (n = 5–6, N = 4), (B) testosterone (n = 5, N = 4), (C) dihydrotestosterone (n = 5–6, N = 4) and the depsidones (D) Unguinol (n = 4–5, N = 3) and (E) Aspergillusidone D (n = 4, N = 3). Data are represented as means ± SEM. No relevant effects (*i.e.* exceeding the variation of vehicle control and statistically different from vehicle control) were seen.

statistically significant reduction in the number of cells in the G0/G1-phase (Fig. 4A). In addition, an increase in the number of MDA-MB-231 cells in the G2/M-phase was seen. The androgens testosterone and dihydrotestosterone did not affect the cell cycle at 50 µM (Fig. 4A), or at concentrations up to 250 µM (data not shown). Exposure to 100 µM Unguinol statistically significantly reduced the number of cells in the S-phase while increasing the number of cells in the G2/M-phase (Fig. 4B). Aspergillusidone D did not statistically significantly affect cell cycle status in MDA-MB-231 cells at 60 µM.

3.4. Effect of doxorubicin, hormones and depsidones on apoptosis in MDA-MB-231 cells

To determine whether the observed cell cycle arrest in the MDA-MB-231 cells by hormones and depsidones could result in apoptosis, the latter effect was determined using the Muse™ Annexin V & Dead Cell Assay kit after a 24-h exposure. Our results show that only the positive control doxorubicin induced apoptosis at the measured concentration (0.5 µM; Fig. 5). The small, yet not statistically significant increase in apoptotic cells after 17β-estradiol exposure may correspond with the small portion of debris seen in the cell cycle arrest assay (Fig. 4A). Even though the depsidones show the potential to induce apoptosis, this was not statistically significantly different from vehicle-treated control cells due to high biological variation.

4. Discussion

This study aimed to investigate the potential of two depsidones, Unguinol and Asperigillusidone D, as possible treatment options for triple-negative breast cancer tumours using MDA-MB-231 cells. Our results confirm the high sensitivity of the MDA-MB-231 cells to anti-tumor effects of doxorubicin that has been observed in earlier studies [28,29]. The proposed mechanism by which doxorubicin induces apoptosis and cytotoxicity are 1) the intercalation into the DNA and the disruption of topoisomerase-II-mediated DNA repair and 2) *via* the generation of free radicals [30]. While doxorubicin is a potent inducer of apoptosis, doxorubicin also induces cardiotoxicity when used to treat breast cancer [6,7]. As chemotherapeutic drugs used to treat hormone receptor-negative breast cancer are of aggressive nature and induce many side effects, novel treatments options are a priority in triple-

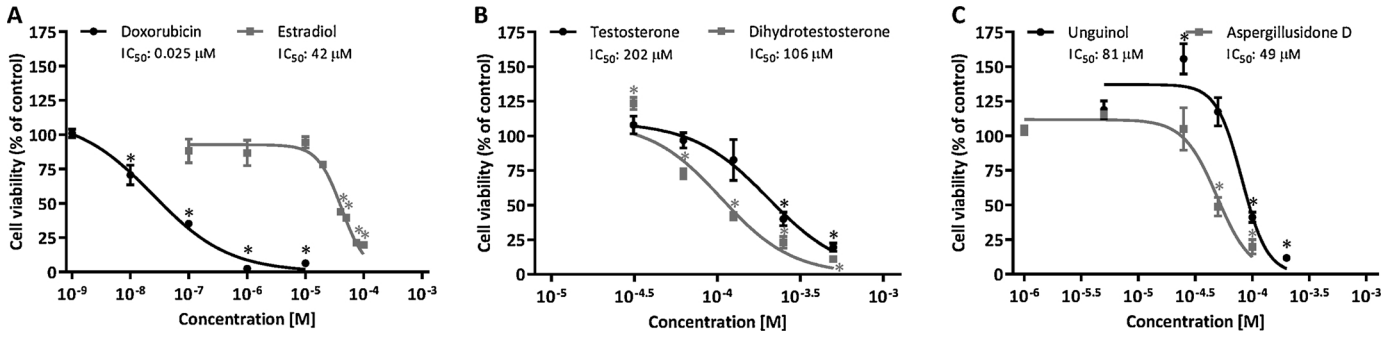

Fig. 3. Cell viability in MDA-MB-231 triple-negative breast tumour cells after a three-day exposure to (A) doxorubicin (n = 4–5, N = 5) and 17β-estradiol (n = 4–5, N = 3–6), (B) testosterone (n = 4–5, N = 4) and dihydrotestosterone (n = 4–5, N = 4) and (C) the two depsidones Unguinol (n = 4–5, N = 3) and Aspergillusidone D (n = 4–5, N = 4). Data are represented as means ± SEM. Relevant effects (*i.e.* exceeding the variation of vehicle control and statistically different from vehicle control) are depicted with * ($p < 0.05$).

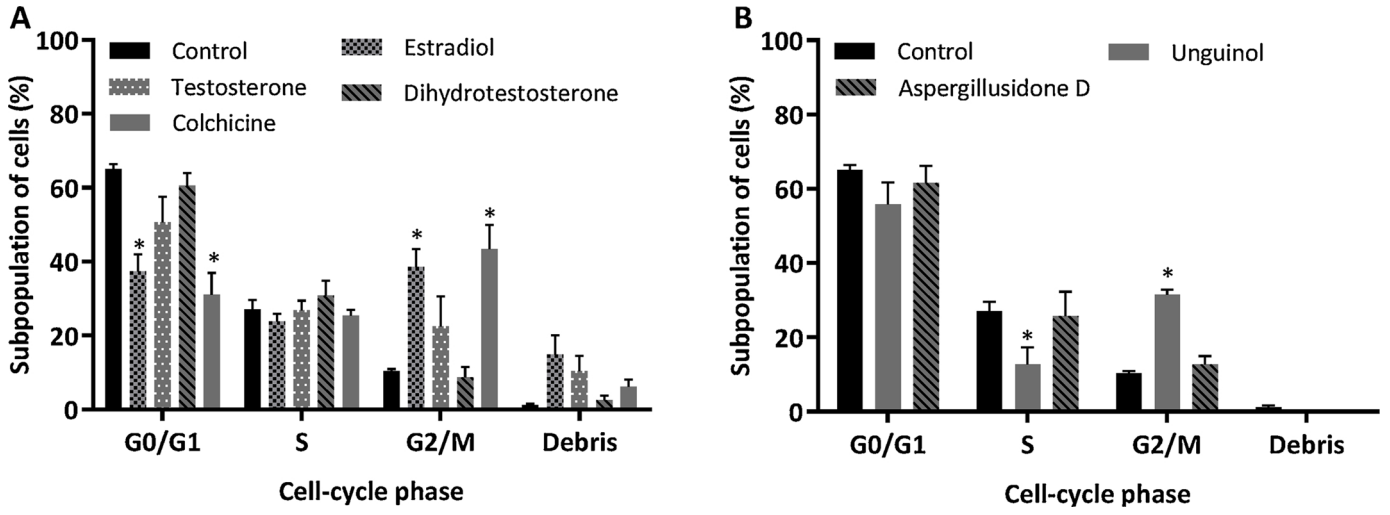

Fig. 4. Cell cycle arrest in MDA-MB-231 triple-negative breast tumour cells induced by (A) vehicle control (0.05% DMSO; n = 1–3, N = 6), colchicine (3 μM; n = 1–3, N = 9–10), 17β-estradiol (50 μM; n = 1–3, N = 9–10), testosterone (50 μM; n = 1–3, N = 6–7), dihydrotestosterone (50 μM; n = 1–3, N = 3) and (B) the two depsidones Unguinol (100 μM; n = 1–5, N = 4) and Aspergillusidone D (60 μM; n = 2–4, N = 3). Data are represented as means ± SEM. Values statistically significantly different from vehicle-treated control cells are depicted with * ($p < 0.05$).

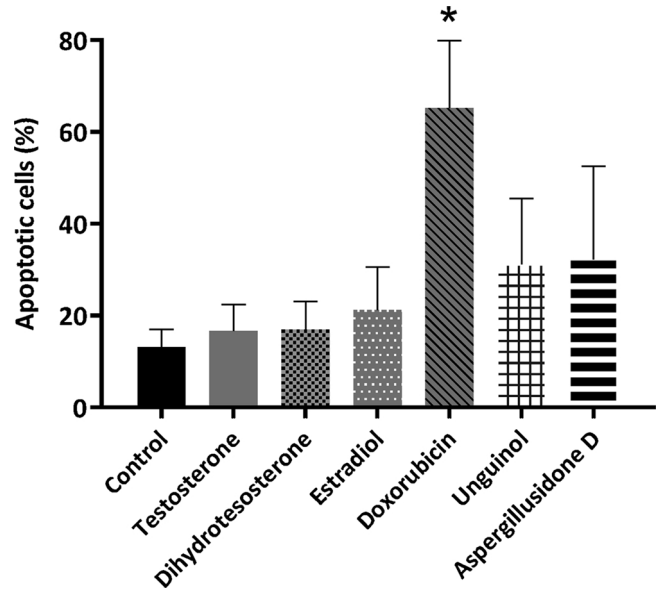

Fig. 5. Apoptosis in MDA-MB-231 triple-negative breast tumour cells induced by vehicle control (0.05% DMSO; n = 2–4, N = 4), doxorubicin (0.5 μM; n = 2–3, N = 4), 17β-estradiol (50 μM; n = 2–3, N = 3), testosterone (50 μM; n = 2–3, N = 3), dihydrotestosterone (50 μM; n = 2–3, N = 3), and the two depsidones Unguinol (100 μM; n = 2–3, N = 2) and Aspergillusidone D (60 μM; n = 2–3, N = 2). Data are represented as mean ± SEM. Values statistically significantly different from vehicle-treated control cells are depicted with * ($p < 0.05$).

negative breast cancer research.

In this study, MDA-MB-231 cells were also exposed to the endogenous hormones 17β-estradiol, testosterone and dihydrotestosterone, to study the effects of the natural tumor microenvironment. 17β-Estradiol did not affect cell proliferation and apoptosis but did induce cytotoxicity and cell cycle arrest (G2/M) at concentrations reported earlier in literature [31–34]. As the MDA-MB-231 cell line does not express the ERα [35], and the role of ERβ in MDA-MB-231 is not well described, effects on cell viability and the cell cycle suggest an ER-independent pathway. It has been brought forward that apoptosis and cell cycle arrest could be induced by 17β-estradiol *via* the inhibition of microtubule assembly by modifying the microtubule polymerization process [36], similar to the effects of colchicine [37].

In line with earlier studies, testosterone and its metabolite dihydrotestosterone did not affect cell proliferation but did reduce cell viability [32,38,39]. 17β-estradiol affected cell viability at concentrations 2-4-fold lower than the androgens. To our knowledge, this is the first study to determine (the lack) of effects of these androgens on apoptosis and cell cycle arrest in MDA-MB-231 cells. This in contrast to the effects observed for androgens in other tissues (*e.g.* testosterone induced apoptosis in mouse ovarian granulosa cells [40] and neuronal cells [41], and cell cycle arrest in coronary smooth muclue cells [42], while dihydrotestosterone induced apoptosis and cell cycle arrest in liver cells [43] and prostate carcinoma cells [44]). The fact that the primary target of these compounds, the androgen receptor (AR), is not present in the MDA-MB-231 cell line most likely explains the absence of effects seen in our apoptosis and cell cycle arrest assays [39]. Cell lines expressing the AR, the MDA-MB-453 and the MDA-MB-231-AR (stably transfected) did show a decrease in cell proliferation and cell growth when exposed to dihydrotestosterone and selective androgen receptor modulators (SARMs) [38,39]. As AR are present in 40–70% of the ER-negative breast cancer tumours, they have become of great interest in developing novel treatments [38,45]. Moreover, previous studies from our lab have shown that dihydrotestosterone, but not testosterone, decreased MDA-MB-231 proliferation when co-cultured with breast adipose fibroblasts [46]. This suggests that the anti-proliferative effects of dihydrotestosterone may partly arise from effects on fibroblasts surrounding breast cancer epithelial cells.

As most cytostatics used for the treatment of triple-negative breast cancer tumours commonly induce early or late-onset cardiotoxicity as a side effect, the search for other, less (cardio)toxic compounds affecting multiple breast cancer targets is ongoing. We showed that both depsidones did not affect cell proliferation at the tested concentrations. However, norstictic acid, a different depsidone, was shown to inhibit the proliferation of six different breast cancer cell lines. For two triple-negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468) the inhibition of proliferation by norstictic acid was seen at concentrations ≥10 μM [47]. As the highest concentration tested in our study was 5 μM, possible effects of Unguinol and Aspergillusidone D at higher concentrations should be included in future experiments.

The potency of Unguinol to induce cytotoxicity was lower than that of Aspergillusidone D. This difference may possibly be caused by the presence of two bromine atoms in Aspergillusidone D compared with

Unguinol, which could enable the formation of halogen bonds [48]. The observed effects on cell viability in our experiments are in good agreement with earlier depsidone studies [49–53]. Our study is the first that investigated the effects of depsidones on other endpoints than cytotoxicity and cell proliferation in MDA-MB-231 cells. We showed that only Unguinol caused cell cycle arrest at concentrations close to the IC_{50} value for cytotoxicity. For this situation, it could be suggested that the observed cell cycle arrest will lead to cell death. This could make Unguinol of potential clinical relevance. As the prolonged arrest of the cells in the G2/M-phase can lead to apoptosis [54], this mechanism was tested for both depsidones. Even though not statistically significant, an increase of 16–18% in the number of apoptotic cells was seen for both depsidones. It is generally recognized that the induction of apoptosis is very useful for the treatment of cancer [55]. Therefore, this possible effect of depsidones should be studied more closely in the future using multiple cell lines and establishing more detailed concentration-effects relationships. In addition, co-exposure and/or alternating treatment with frequently used cytotoxic treatments for triple-negative breast cancer or (novel) promising phytochemicals with anti-cancer properties like ethyl *p*-methoxy cinnamate [56], resveratrol [57] or polyphenolic extracts from grape stems [58] should be included as this could possibly enhance anti-cancer actions and reduce treatment resistance and side effects.

As the depsidones affected cell viability and cell cycle arrest with different potencies in our study, multiple mechanisms of action could be involved. At the moment, detailed insights into the mechanism of action of depsidones in breast cancer tumour cells are limited. Earlier studies indicate that depsidones can fragment genomic DNA, increase caspase-3 activity, increase ROS generation [55] and inhibit proto-oncogenic tyrosine kinase (c-Met) activity [47]. Moreover, it has been observed that depsidones target tumour cell lines but not non-tumour cell lines [47], supporting the applicability of depsidones as options for treatment.

Naturally occurring depsidones have been shown to exhibit anti-tumor properties in hormone receptor-positive breast cancer cells. In the present study, we showed that this group of compounds may be suitable for anti-cancer treatment of hormone triple-negative breast cancer as well. The depsidones tested in this study, Unguinol and Aspergillusidone D, induced cytotoxicity and apoptosis at μM concentrations in MDA-MB-231 cells. Moreover, Unguinol induced cell cycle arrest. Considering that cytotoxicity, apoptosis and cell cycle arrest are all relevant features in anti-cancer treatment, this group of compounds is of potential clinical relevance in the treatment of triple-negative breast cancer tumours. Future studies should be performed to investigate underlying anti-cancer mechanisms and potential side effects of these depsidones.

All authors declared no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

Declaration of Competing Interest

All authors declared no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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