Depsidones inhibit aromatase activity and tumor cell proliferation in a co-culture of human primary breast adipose fibroblasts and T47D breast tumor cells

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ABSTRACT

Naturally occurring depsidones from the marine fungus Aspergillus unguis are known to have substantial anticancer activity, but their mechanism of action remains elusive. The purpose of this study was to examine the anti-aromatase activity of two common depsidones, unguinol and aspergillusidone A, in a co-culture system of human primary breast adipose fibroblasts and hormonal responsive T47D breast tumor cells. Using this in vitro model it was shown that these depsidones inhibit the growth of T47D tumor cells most likely via inhibition of aromatase (CYP19) activity. The IC50 values of these depisidones were compared with the aromatase inhibitors letrozole and exemestane. Letrozole and exemestane had IC50 values of respectively, 0.19 and 0.14 μM, while those for Unguinol and Aspergillusidone A were respectively, 9.7 and 7.3 μM. Our results indicate that among the depsidones there maybe aromatase inhibitors with possible pharmacotherapeutical relevance.

1. Introduction

Depsidones are secondary metabolites mostly found in lichens, but also in some higher plants, where they play a role in the protection against insects and microbes or sun light. Depsidones are esters which compose of polyphenolic depsides and cyclic ethers, but the chemical structure of the depsidones formed is highly dependent on the type of fungus and its environment [1]. Generally, the chemical structure of depsidones resembles prostaglandins and leukotrienes in humans, which makes these compounds may have beneficial health effects in humans. Indeed, several studies have already reported biological activities of these naturally occurring depsidones like anti-proliferative actions [2–4], antimalarial and cytotoxic properties [5], antibacterial activity against the multidrug-resistant Staphylococcus aureus [6], radical scavenging, antioxidant activities and antifungal activities [7–9]. On the other hand, because of their structural similarity, leukotriene depsidones may induce allergic reactions in humans [10]. Also, apoptosis or inflammatory processes that involve free oxigen radicals by depsidones have been reported to cause cytotoxicity in rat hepatocytes [11] and rat thymocytes [12]. More recently, in vitro studies from our laboratory indicate that some depsidones are potent inhibitors of the aromatase enzyme (CYP 19) [7,9]. The aromatase enzyme is responsible for the conversion of androgens into estrogens and is used as a therapeutic target for breast cancer treatment [13,14]. In view of the above biological properties of these depsidones novel cancer treatments may arise. In this respect, the inhibition of aromatase activity may be especially relevant for treatment of estrogen-dependent tumors, such as breast tumors.

Aromatase inhibitors, such as exemestane and letrozole, are currently used as hormonal therapy in estrogen positive postmenopausal breast cancer patients [15]. To detect inhibitory properties of aromatase, studies are frequently performed using relatively simple in vitro models such as human placental tissue in which radiolabeled androgens are used as substrates [16]. Other methods such as HPLC separation with UV detection [17] and recombinant enzyme systems are often less sensitive and laborious [18]. Stresser et al. developed a high throughput
screening method using the fluorometric substrate O-benzyl fluorescein benzyl ester (DBF) as a substrate for microsomal aromatase activity [19]. This method was also found suitable for detecting aromatase inhibition in our present co-culture study. In our previous study, we used a more realistic in vitro breast cancer model that consists of a co-culture of primary human breast fibroblasts with hormonal positive T47D breast cancer cells to study effects on aromatase activity [20]. In this co-culture model, paracrine interactions between various cell types are implemented leading to a more physiologically relevant test model to assess local effects of compounds on aromatase activity and subsequent (anti)tumor effects.

The purpose of this co-culture study was to examine the inhibitory properties of the common depsidones unguinol and aspergillusidine A, which are isolated from the marine-derived fungus A. unguis CR1282-03, on aromatase activity in primary human breast fibroblasts and its subsequent effect on the proliferation of T47D breast tumor cells. The results were compared with two clinical relevant aromatase inhibitors, letrozole and exemestane.

2. Materials and methods

2.1. Depsidones

Depsidones were isolated from Aspergillus unguis CR1282-03, a fungus isolated from the marine environment by the laboratory of natural products from the Chulaborn Research Institute (Bangkok, Thailand) [2,7]. For our present study two depsidones, unguinol (UNG) and aspergillusidine A (ASP-A), were selected. This was based on a preliminary study using the microsomal placental assay, which indicated inhibitory properties for aromatase [7]. Structures of UNG and ASP-A are shown in Fig. 1A and B. Range finding studies in our laboratory indicated cytotoxicity of UNG and ASP-A in the mM range [2,7], which is significantly higher than concentrations used in our present co-culture study. For comparison the IC50 values of letrozole and exemestane were also determined under similar experimental conditions (See Fig. 1C and D). Cytotoxic effects of letrozole and exemestane have been reported earlier to be in the mM range and therefore concentrations in our co-culture experiments did not exceed 0.25 μM.

2.2. Breast cancer cell culture and incubation

The T47D cell line was obtained from ATCC (Rockville, MD, USA). These cells were grown in culture medium comprising of RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% l-glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution. Seeding concentration was 5 × 10^4 cells/ml and cells were cultured at 37 °C in a humidified atmosphere with 5% CO2 until 80–90% confluent.

2.3. Primary breast adipose fibroblasts (BAFs)

After written informed consent, BAFs were obtained from three breast cancer patients, who attended the Bamrasnaradura Infectious Disease Institute (Nonthaburi, Thailand) for modified radical mastectomy. The research protocol was approved by the Medical Ethical Committee of the Bamrasnaradura Infectious Disease Institute. About 5–10 g of macroscopically normal breast tissue was collected, while the remaining tissue was sent for routine pathological examination. Directly after the tissue was obtained via surgery, it was stored in a 4 °C saline solution (0.9% NaCl) and immediately transported to our laboratory. BAFs were isolated from breast tissue as described earlier [21,22]. These BAFs were cultured as adherent cultures in RPMI 1640-medium w/o phenol red (Gibco/Invitrogen 11835) supplemented with Pen/Strep (Invitrogen 15140), FCS (Invitrogen 10270) and Insulin 10^{-5} M (144 mg/25 ml) (sigma I-5500) at a seeding concentration of 5 × 10^4 cells/ml. The cells were maintained in a humidified atmosphere at 37 °C with 5% CO2. BAFs were sub-cultured if cell cultures in the bottles were confluent, which was usually once a week.

2.4. Breast cancer cells in co-culture with BAFs

Approximately three weeks after isolation, fibroblasts were used to establish a co-culture together with the T47D tumor cells [20,22,23]. On day one, BAFs were plated at a density of approximately 4 × 10^3 cells/well in a 96-well plate for proliferation or 5 × 10^3 cells/ml in a 25 cm² flask for gene expression and grown in culture medium as described above. The day after, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on the assay medium in which heat inactivated FCS was replaced with heat inactivated, charcoal/dextran-treated FCS (Hyclone, SH30068.03). On day 4, BAFs were washed with PBS and the T47D cells were trypsinized and seeded on top of the BAFs at a density of 4 × 10^3 cells/well for proliferation or 2.5 × 10^5 cells/flask for gene expression. On day 5, fresh assay medium was added to the co-cultures after which the cells were exposed to the test compounds for 96 h [24]. Final solvent concentration was 0.1% v/v in the medium. Initial concentrations were 1, 5, and 10 nM for testosterone, 50 nM for exemestane, 30 nM for letrozole, and 2 μM for depsidones to screen for aromatase inhibition in the presence of testosterone. In this co-culture model testosterone is converted by aromatase in the BAFs to estradiol, which in turn induces cell proliferation in the estrogen positive T47D tumor cells.

2.5. Cell proliferation

Cell proliferation was determined after treatment with testosterone by performing an MTT assay as described earlier [21]. After 96 h of exposure to the test compounds, 1 ml of MTT (5 mg MTT/ml in PBS) was added for 4 h. Next, the medium was aspirated and 100 μl of DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 550 nm using 96-well plates in a Spectramax plus 384 microplate reader (Molecular Devices, California, USA).

2.6. Modified high-throughput screen to identify inhibitors of Aromatase (CYP19) [19]

Aromatase activity was measured by the conversion of the fluorometric substrate O-benzylfluorescein benzyl ester (DBP) as described earlier [19] with the following minor modifications. Assays were conducted in 96-well microtiter plates (Catalog No. 3915, Corning Costar, Cambridge, MA) using a 200 μl volume containing a serially
dilution of 1:2 of UNG, ASP-A, exemestane or letrozole. DBF was obtained from Gentest Corporation (Woburn, MA). In this method the metabolite production of DBP by aromatase is linear in time up to 30 min and 2 μM DBP, which was used to determine IC_{50} values of the test compounds. The enzymatic reaction was terminated by adding 40 μl 2 N NaOH. To develop adequate signal to background ratio, the 96-well plates (with lid) were incubated for 2 h at 37 °C. Fluorescence in each dividual well was measured in top-read mode using a FLUOstar Model 403 fluorescence plate reader (BMG LabTechnologies, Inc., Durham, NC). DBF metabolite concentrations were measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.7. Analysis of gene expression

After the cells were treated total RNA was extracted. RNA was reverse-transcribed into cDNA using the Qiagen high capacity cDNA reverse-transcription kit (Qiagen, Texas, USA). The gene expression of pS2 and Ki-67 was performed using a Roche-Light-cycler-480 one step RT PCR kit (Roche, Indiana, USA). Relative quantification of gene expression was expressed as the ratio of the intensity of the target gene to the housekeeping gene beta-actin. The RT-PCR conditions for each gene are described below.

2.8. RNA isolation and gene expression

Total RNA was isolated using RNeasy® mini kit (Qiagen, Texas, USA) according to the manufacturer’s instruction. Cells were centrifuged and lysed with RLT buffer and β-mercaptoethanol. One volume of 70% ethanol was added and this was vigorously mixed. The sample was transferred to a RNeasy spin column, centrifuged at 12,000g for 15 s, and then 350 μl washing buffer RW1 was added on the column and again centrifuged at 12,000 g for 15 s. The 80 ul of DNase I reaction mixture was directly added on the membrane of the column and incubated for 15 min. Thereafter, the column was washed with RW1 buffer and RPE buffer twice. Bound RNA was eluted with 40 ul of RNase-free water and centrifuged at 12,000g for 1 min. The purification and concentration of total RNA were measured by the ratio of absorbance at 260/280 and 260/230 NM using Nanodrop (Nanodrop Technologies, Inc., Delaware, USA).

Obtained RNA was stored at −20 °C in aliquots of 10 ng/μl. The reaction mixture contained 0.1 μM of primers, 1X Quantitect SYBR Green RT-PCR Master mix, 0.2 μl QuantiTect RT mix and 2 μl template RNA (10 ng/μl) in a total volume of 20 μl. The mixture was reverse transcribed to cDNA at 50 °C for 20 min. After reverse transcription, the PCR reaction was initiated by heating at 95 °C for 15 min, then followed by denaturation at 95 °C for 10 s, annealing at 57 °C for 25 s, and extension at 68 °C for 30 s and acquisition at 82 °C for 5 s for 45 cycles. Primers coding for the estrogen-responsive pS2 gene were used as described earlier by Lee et al. [25,26]. Primers for Ki-67 amplification were designed in our laboratory as described previously [23]. Expression of β-ACTIN (forward: 5′-TCTACAATGAGCTGCG-3′ and reverse: 5′-AGGTAGTCAGCTAGGT-3′) was used as a reference housekeeping gene. All primers were run through the National Center for Biotechnology Information (NCBI) blast (nucleotide nonredundant database) to confirm specificity. The efficiency was determined for a dilution range of cDNA and primers were only used with an efficiency between 95% and 105%. After each RT-qPCR, a melt curve was run to ensure that primer-dimers and other non-specific products were omitted.

2.9. Data analysis

Each experiment and each concentration were performed in triplicate. Data were analyzed using Microsoft Excel and IC_{50} values calculated by interpolation. Means and standard deviations were calculated of all nine values (n = 9). The statistical significance of differences of the means were calculated using the Student’s t-test or one-way ANOVA. Differences were considered statistically significant if P < 0.05.

3. Results

3.1. Exposure of mono and co-cultured T47D cells with BAFs to testosterone

Firstly, aromatase activity and cell proliferation were determined in a co-culture of BAFs and T47D cells to validate the performance of the co-culture model. BAFs were obtained from one patient, since it has been established that aromatase activity can vary largely between individuals [21,23]. Adding testosterone up to 10 nM increased proliferation of T47D cells in the co-culture up to 30%. In contrast, adding testosterone to a mono-culture of T47D cells caused a maximum reduction in cell growth of 50% at 10 nM (p < 0.01) (See Fig. 2). These observations confirm the aromatization of testosterone to estradiol in the co-culture of T47D and BAFs, which is followed by a subsequent increase in estrogen-dependent cell proliferation of the tumor cells. Furthermore, the reduction of cell proliferation in the mono culture of T47D cells by testosterone confirms our earlier observations that androgens can reduce the proliferation of androgen receptor positive breast cancer cell lines like that of the T47D cells [23]. The inhibition of cell proliferation of T47D cells in co-culture with BAFs by the different test compounds at different concentrations of testosterone was also studied. As could be expected the aromatase inhibitors letrozole and exemestane inhibited T47D cell proliferation at
concentrations of respectively 30 and 50 nM (See Fig. 3A and B). At concentrations of 2 μM UNG and ASP-A inhibition of T47D cell proliferation in these co-cultures was observed, albeit at approximately two orders of magnitude higher concentrations than those of letrozole and exemestane (see Fig. 3C and D). These results indicate that UNG and ASP-A may act as aromatase inhibitors in these T47D and BAF co-cultures. In the subsequent experiments the relative potency of these compounds as aromatase inhibitors were studied in further detail for confirmation.

3.2. Aromatase inhibition in co-cultures of T47D cells and BAFs

The inhibitory potency of letrozole, exemestane, UNG and ASP-A on aromatase activity was determined at a fixed DBP concentration of 2 μM. A concentration-dependent inhibition of aromatase was observed for all four compounds. IC_{50} values for letrozole, exemestane, UNG and ASP-A were 0.19 μM (95% CI; 0.13–0.28 μM), 0.14 μM (95% CI; 0.11–0.18 μM), 9.73 μM (95% CI; 7.31–12.96 μM) and 7.26 μM (95% CI; 4.99–10.57 μM), respectively. Concentration – response relationships for these compounds are shown in Fig. 4A–D.

3.3. pS2 and Ki67 gene expression in co-cultures of T47D cells and BAFs

When a co-culture of T47D cells and BAFs was exposed to testosterone only (1, 5, 10 nM), a concentration dependent increase in the expression of pS2 and Ki67 mRNA was observed (p < 0.01) (Fig. 5A and B). The induction of both cell proliferation markers was considered to be induced by estradiol production via aromatase in the BAFs. To confirm this, the T47D and BAF co-cultures were exposed to 1, 5 or 10 nM testosterone in combination with the aromatase inhibitor exemestane (50 nM). Adding exemestane completely negated the induction of pS2 and Ki67 mRNA expression, which confirmed the functional role of aromatase in this estradiol production. Similarly, UNG and ASP-A (2 μM) fully negated the testosterone-induced expression of pS2 and Ki67 mRNA, which again confirms their role as aromatase inhibitors.

4. Discussion

In this study, we clearly show that the naturally occurring despidones unguinol and aspergillusidone A can effectively inhibit aromatase activity and subsequently reduce estrogen-dependent breast cancer cell proliferation in an in vitro co-culture model with T47D breast cancer cells and primary BAFs.

4.1. Physiological relevance of the co-culture system

This co-culture system consist of T47D breast tumor cells in combination with breast adipose tissue fibroblasts (BAFs). In our earlier studies, we established that this T47D cell line expresses both the estrogen α and androgen receptors and is very suitable for use in a co-culture with BAFs to mimic the in vivo breast cancer situation [23,27]. The role of steroid hormones in breast tumors is complex due to the various paracrine interactions between different cell types in the breast.
Fig. 4. Inhibition of aromatase activity in co-cultures of T47D cells and human breast adipose fibroblasts by letrozole (a), exemestane (b), UNG (c) and ASP-A (d). Data are expressed as mean DBP intensity as marker for aromatase activity compared to vehicle-treated control cells ± SD (N = 3).

Fig. 5. Effect of testosterone, exemestane, UNG and ASP-A on pS2 (A) and Ki67 (B) mRNA expression in co-cultures of T47D cells and human breast adipose fibroblasts. Data are expressed as means ± SD (n = 9). * Statistically significantly different from vehicle-treated control co-cultures (p < 0.001).
tissue. We and others have previously shown that (part of) the intercellular communication between the tumor cells and surrounding BAFs remains operational in our co-culture model [21,28,29]. Therefore, the effects studied in the co-culture model used in present study have more physiological relevance and predictive value compared to effects a monoculture of breast tumor cells in vitro. For estrogen dependent breast tumors the presence of surrounding BAFs is especially relevant, as the local estrogen production is situated in BAFs due to the presence of the aromatase enzyme. In several earlier studies with these breast co-cultures we have shown their relevance for studying natural compounds like androgens, melatonin and resveratrol [20–23]. Most co-culture studies have focused on estrogen dependent tumors. However, a substantial number of breast tumors are not hormonal responsive and for these clinical treatment options are very limited [30–33]. At present, little information is available about the paracrine interactions and intercellular communications between non-hormonal responsive (triple negative) breast tumor cells and BAFs. In the quest for finding new compounds with anti-tumor properties for these non-hormonal responsive breast tumors these co-cultures may offer a more advanced in vitro testing system.

### 4.2. Depsidones as aromatase inhibitors

Our institute has already published several studies on depsidones that were isolated from A. ungii CR2282-03, which had anti-aromatase activity [2,9,34]. These previous studies with A. ungii-derived ASP-F, D, E and Unguinol showed that inhibition of aromatase activity by these depsidones was in the same order of magnitude, ranging between 0.5 and 1 μM [7]. This suggests that the differences in chemical structure with respect to halogen substitution and hydroxyl placement, have no apparent effect on the aromatase inhibitory potency of the depsidones tested. Although ASP-A was not tested in that study, the IC50 for aromatase inhibition for unguiolin was 0.6 μM compared with 9.7 μM in present study. This difference can most likely be attributed to the difference in experimental set-up; the study of Sureram et al. used recombinant human aromatase enzyme, while in this study primary human breast cells were used. In present study, the bioavailability of the tested depsidones might have been affected by metabolism or active excretion from the cells. Moreover, in present study, the aromatase inhibiting potentials of UNG and ASP-A were two orders of magnitude lower than the clinically used therapeutic exemestane. Yet, the aromatase inhibiting properties occurred at non-cytotoxic concentrations, which is relevant to assess with respect to breast cancer therapeutic. Other depsidones; e.g. fungal-derived norstictic acid and plant-derived phomopsidone A and atrovirisidone B have been shown to cause cytotoxicity in breast cancer cell lines. Norstictic acid inhibited MDA-MB-231 proliferation by inhibition of proto-oncogenic tyrosine kinase (c-Met) activity [35]. Phomopsidone A, which has the structure similar to breast cancer drug named Taxol, also inhibited the proliferation of breast cancer cell line in vitro [36]. Atrorivisodone B displayed cytotoxicity in MCF-7 cells with an IC50 value of approximately 23 μM [37]. Clearly, there are more types of depsidones with bioactive properties that might be relevant for breast cancer research. To determine the clinical relevance of our findings with respect to aromatase inhibition, clearly a more toxicological and pharmacological comparison between these depsidone and standard anti-aromatase is needed. These studies should then also address possible adverse effects of depsidones; e.g. caused by cytotoxicity at the higher dose levels.

### 5. Conclusion

Our results demonstrate that in a co-culture system of estrogen-dependent T47D breast tumor cells and primary breast adipose fibroblasts two depsidones, unguinol and aspergillusidone A, can act as aromatase inhibitors leading to inhibition of tumor cell proliferation. The multiple anti-cancer activities of the depsidones warrant further studies for their potential clinical applications.

### Conflict of interest statement

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

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