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Article

Pseudopterosin Inhibits Proliferation and 3D Invasion in Triple Negative Breast Cancer by Agonizing Glucocorticoid Receptor Alpha

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Abstract: Pseudopterosin, produced by the sea whip of the genus *Antillogorgia*, possesses a variety of promising biological activities including potent anti-inflammatory effects. However, few studies examined pseudopterosin in the treatment of cancer cells and, to our knowledge, the ability to inhibit triple negative breast cancer (TNBC) proliferation or invasion has not been explored. Thus, we evaluated the as yet unknown mechanism of action of pseudopterosin: Pseudopterosin was able to inhibit proliferation of TNBC. Interestingly, analyzing breast cancer cell proliferation after knocking down glucocorticoid receptor α (GRα) revealed that anti-proliferative effects of pseudopterosin were significantly inhibited when GRα expression was reduced. Furthermore, pseudopterosin inhibited invasion of MDA-MB-231 3D tumor spheroids embedded in an extracellular-like matrix. Remarkably, the knockdown of GRα in 3D tumor spheroids revealed increased ability of cells to invade the surrounding matrix. In a co-culture, encompassing peripheral blood mononuclear cells (PBMC) and MDA-MB-231 cells, production of interleukin 6 (IL-6) and interleukin 8 (IL-8) significantly increased compared to monoculture. Notably, pseudopterosin proved to block cytokine elevation, representing key players in tumor progression, in the co-culture. Thus, our results reveal pseudopterosin treatment as a potential novel approach in TNBC therapy.

Keywords: Pseudopterosin, triple negative breast cancer, glucocorticoid receptor alpha, dexamethasone, cell proliferation, 3D invasion, tumor spheroid, co-culture, interleukin 6, interleukin 8.

1. Introduction

Breast cancer is still the most common malignancy in woman with one million cases annually worldwide[1]. Of these, approximately 15% belongs to the triple-negative (ER$^-$/PR$^-$/HER2$^-$) breast cancer (TNBC). TNBC represents the most aggressive breast cancer type, characterized by high proliferation rate, a pronounced potential to metastasize and a shorter survival rate[2–4]. Furthermore, TNBC lacks effective therapies available for other breast cancer subtypes underlining the significant unmet medical need for identifying novel targets and developing innovative drugs.

The tumor microenvironment is increasingly recognized as a major regulator of carcinogenesis. In breast cancer, tumor associated macrophages (TAMs), representing over 50% of the tumor mass, enhance proliferation and metastasis as well as resistance to chemotherapy by activation of the transcription factor nuclear factor κB (NF-κB), a key factor in regulating inflammatory responses[5,6]. High expression levels of the NF-κB target genes interleukin 6 (IL-6) or interleukin 8 (IL-8) secreted by macrophages can be correlated with advanced growth of TNBC and poor prognosis[7].

The pseudopterosins, a family of 31 known related diterpene glycosides, are produced by the sea whip *Antillogorgia elisabethae* (formerly named *Pseudopterosin elisabethae*)[8]. Striking biological

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activities have been described ranging from anti-inflammation[9–11], wound-healing[10,11], analgesia-reducing[9,12,13] to neuromodulation[14]. In contrast, to date, little is known regarding anti-tumor effects of pseudopterosin, where only one derivative showed moderate cytotoxic effects on ER^+ breast cancer cells and non-small-cell lung cancer cells[15].

Previously, we have described the potential of pseudopterosin as a novel immune modulator in TNBC acting via NF-κB inhibition and subsequent blockade of cytokine secretion[16]. Moreover, we identified inhibitory capabilities of pseudopterosin on the NF-κB signaling pathway by agonizing the glucocorticoid receptor α (GRα)[16]. Accordingly, there is evidence that NF-κB and GRα can physically interact and heterodimerize in breast cancer[17]. By binding other transcription factors such as NF-κB, GRα can either transactivate or -suppress its target genes[18].

Although glucocorticoids (GCs) are frequently used to relieve symptoms of cancer treatment related side effects, contradictory effects on breast cancer progression upon GC treatment and with respect to GRα expression have been described[19–21]. High expression levels of GRα in ER^- breast cancer might be associated with drug resistance resulting in an unfavorable clinical outcome[22–24]. In contrast, a recent analysis demonstrates improved survival independent of the ER status in breast cancer patients receiving GC combined with adjuvant anthracycline-based chemotherapy[25]. Thus, in the current study we further elucidated the role of GRα in TNBC progression, thereby focusing on pseudopterosin as a novel agent for breast cancer therapy.

2. Results

2.1. Pseudopterosin Inhibited Proliferation of Triple Negative Breast Cancer Cells

In our previous work we identified the natural product pseudoperosin as a novel inhibitor of NF-κB signaling[16], one key pathway in controlling progression of TNBC. As NF-κB is known to regulate various processes in cancer progression such as proliferation, angiogenesis or invasion[26–28], the aim of the current study was to further characterize the pharmacological properties of pseudopterosin. First, we investigated a pseudopterosin extract (PsA-D) regarding its effect on breast cancer cell proliferation in MDA-MB-231 cells. To remain within a non-toxic concentration range of PsA-D (IC_{50} values of cell viability for PsA-D after 24 hours or 48 hours of treatment were 31.4 µM and 32.2 µM, respectively; Supplemental Fig. S1A/B), 7.5 and 15 µM of PsA-D were chosen to evaluate anti-proliferative effects (Fig. 1A). As expected, MDA-MB-231 cells treated with DMSO showed a high proliferation rate, represented by a confluency of 78% after 48 hours (Fig. 1A). Notably, a concentration of 15 µM of PsA-D was able to reduce proliferation significantly after 24 hours by 1.9 fold and after 48 hours by 1.6 fold compared to DMSO control (Fig. 1B and 1C). Furthermore, preliminary data indicate that pseudopterosin-induced reduction of proliferation is not pERK dependent (data not shown), which is a key regulator for cell proliferation in principle[29].

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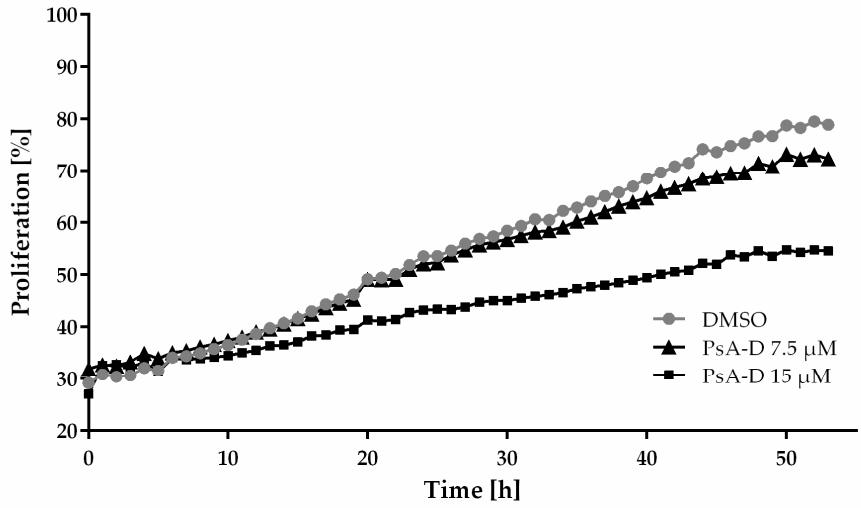

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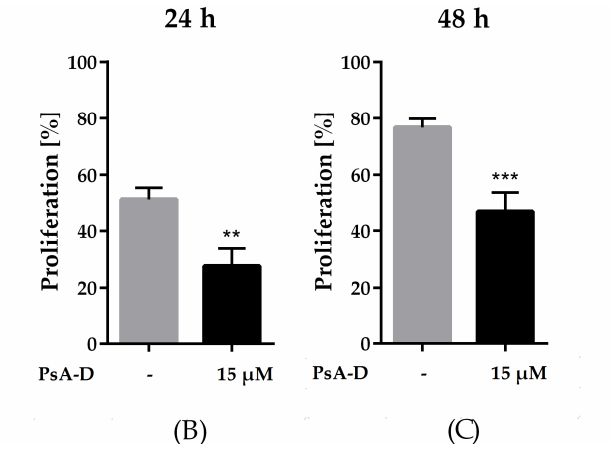

(B) (C)

Figure 1. Pseudopterosin inhibited proliferation in triple negative breast cancer cells. (**A**) Proliferating cells were imaged every hour over a time range of 50 hours with the IncuCyte® ZOOM. Confluency of cells was determined with IncuCyte® software indicated as proliferation in percentage. Cells were treated with either 7.5 µM (triangle) or 15 µM (square) of PsA-D. (**B-C**) Inhibition of proliferation is shown at selected time points of 24 and 48 hours compared to DMSO control, respectively. The data represent means of three independent experiments. Error bars were calculated using ±SEM. P-values were calculated against DMSO control. Two stars represent a significance of $p<0.01$ and three stars represent a significance of $p<0.001$.

2.2. Glucocorticoid Receptor Alpha Expression is Essential for Anti-Proliferative Effects of Pseudopterosin

In our previous work we hypothesized pseudopterosin to act as an agonist of the glucocorticoid receptor alpha (GRα)[16]. Subsequently, when downregulating GRα, pseudopterosin failed to inhibit NF-κB target gene expression. Thus, to further explore the role of GRα in the mode-of-action of pseudopterosin, we analyzed the effect of a GRα knockdown on breast cancer cell proliferation. After 72 and 85 hours, treatment with PsA-D inhibited proliferation in non-coding siRNA (nc siRNA) transfected cells by 2 fold, respectively (Fig. 2A and Supplementary Fig. S2). Importantly, in siGRα transfected cells, PsA-D lost its anti-proliferative effect (Fig. 2A). Efficiency of the GRα knockdown (up to 88%) is exemplified in Fig. 2B. In conclusion, our data suggests that GRα expression might be crucial for the anti-proliferative effects of PsA-D.

Notably, treatment with the marked GRα ligand dexamethasone showed less potency in reducing proliferation: after 48 hours, PsA-D resulted in a 21% proliferation decrease, whereas 100

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nM dexamethasone reduced proliferation by 15% compared to DMSO, respectively (Fig. 2C). After 72 hours, PsA-D treatment diminished proliferation by 20%, whereas treatment with 100 nM dexamethasone reduced the proliferation rate by only 9% (Fig. 2D).

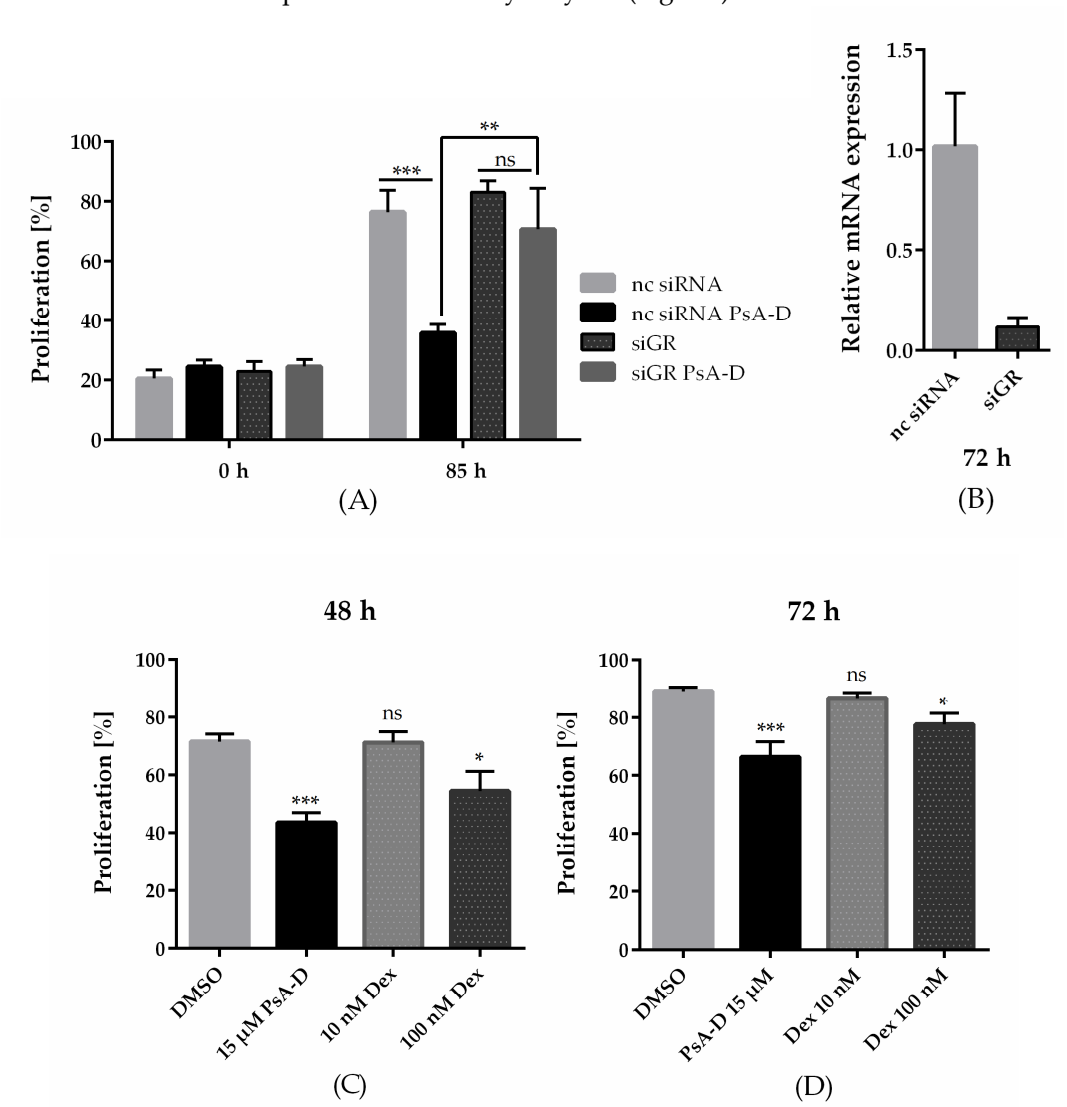

Figure 2. Pseudopterosin failed to inhibit breast cancer cell proliferation after knockdown of the glucocorticoid receptor alpha (GRα) and inhibited proliferation of MDA-MB-231 more efficaciously than dexamethasone (Dex). (**A**) Knockdown of GRα was done with the Lonza Nucleofector 2b device on day one. On day two, the cells were seeded and proliferating cells were imaged with the IncuCyte® ZOOM every hour over a time range of five days. Cell proliferation was determined with IncuCyte® software indicated in percentage. Cells were treated with a concentration of 15 µM of PsA-D. (**B**) After knockdown of GRα, expression of GRα reduced up to 88.3%, which was confirmed by qPCR analysis at 72 hours. (**C-D**) PsA-D inhibited proliferation after 48 and 72 hours more efficaciously than dexamethasone. The data represent means of three independent experiments. Error bars were calculated using ±SEM. Three stars represent a significance of $p<0.001$ and two stars of $p<0.01$.

2.3. Pseudopterosin Inhibited Invasion into 3D Matrix

Breast tumors harbour many devastating charactersitics resulting in poor prognosis of patients: high proliferation rate and high histological grade. Furthermore, genetic and epigenetic alterations enable breast cancer cells to migrate and invade the surrounding tissue via a process known as epithelial-to-mesenchymal transition (EMT)[30]. To explore the effcts of pseudopterosin on the

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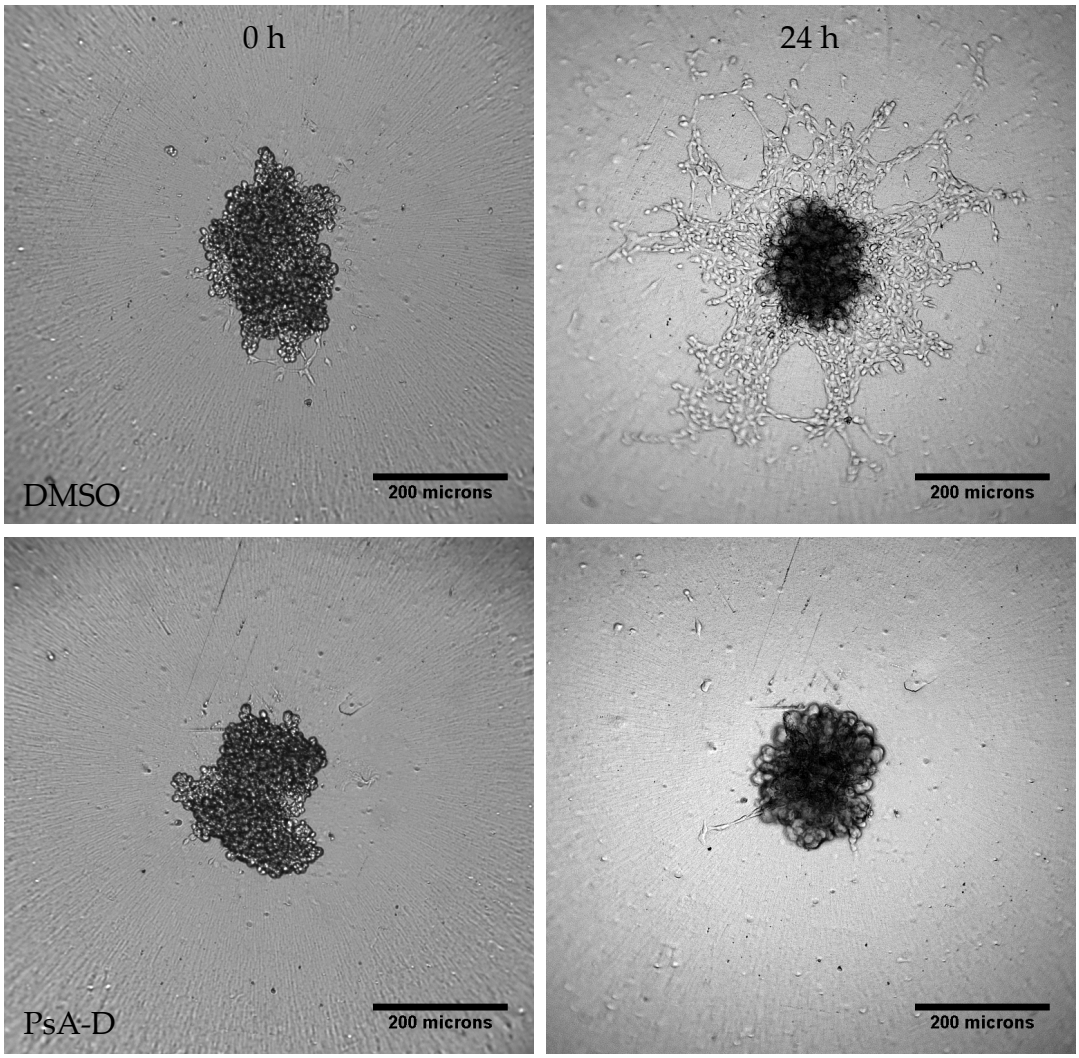
invasiveness of MDA-MB-231 cells, we developed a 3D invasion assay, where the cancer cells form a micro-tumor spheroid embedded in extracellular matrix. In the presence of DMSO, the cells immediately started to invade into the 3D matrix by partly disassembling the spheroid core (Fig 3A). In contrast, treatment with PsA-D inhibited the invasion of single cells into the matrix significantly. Furthermore, disassemblance of the sperhoid core was blocked (second row). After 24 hours, the invasive area was reduced significantly by 59%, after 48 hours by 53% and after 72 hours by 73% (Fig. 3B-D). In this experiment we verified the inhibitory properties of pseudopterosin in a 3D assay on TNBC progression, thereby hinting at a better prediction for future *in vivo* tumor models with this natural product.



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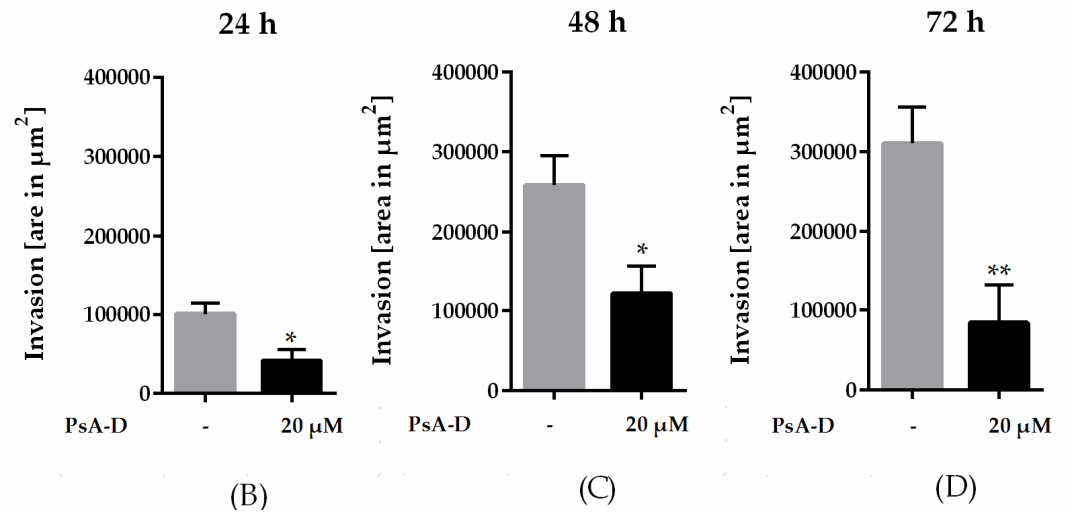

Figure 3. Pseudopterosin inhibited invasion into a 3D matrix. (**A**) Representative images of invasion of cells into a 3D matrix at 24 hours' time point. Cells were imaged with IncuCyte® ZOOM over a time range of three days. 3*10³ cells per well were seeded into ULA round bottom plates and spheroids were formed for 72 hours. Scale bars in black show 200 microns in length. (**B-D**) The bar diagrams show three different time points representing six independent experiments. Spheroids were treated with a concentration of 20 µM of PsA-D. Error bars were calculated using ±SEM. P-values were calculated against control (CTRL). Two stars represent a significance of $p<0.01$ and one star represents a significance of $p<0.05$.

2.4. Down-Regulation of Glucocorticoid Receptor Alpha Expression Increased Invasiveness in TNBC

Recently, the clinical use of glucocorticoids (GC) is discussed controversially, due to extensive side effects, chemotherapy resistance and survival of cancer cells[21,23,31]. However, recent literature indicates the beneficial effects of GCs to be strongly dependent on the tumor entity: survival in patients receiving GC combined with anthracycline-based chemotherapy was improved[25]. In this context we further investigated the role of GRα in the invasiveness of MDA-MB-231 micro-tumor spheroids (Fig. 4A). The efficiency in GRα knockdown is represented by a reduction of 94% (Fig. 4C). After 72 hours, the spheroids transfected with siGRα showed a significant increase in invasion by 27% compared to nc siRNA (Fig. 3B). In conclusion, the knockdown of GRα led to an elevation of invasiveness in MDA-MB-231 cells, hinting at the potential of GRα agonists like pseudopterosin in diminishing TNBC progression

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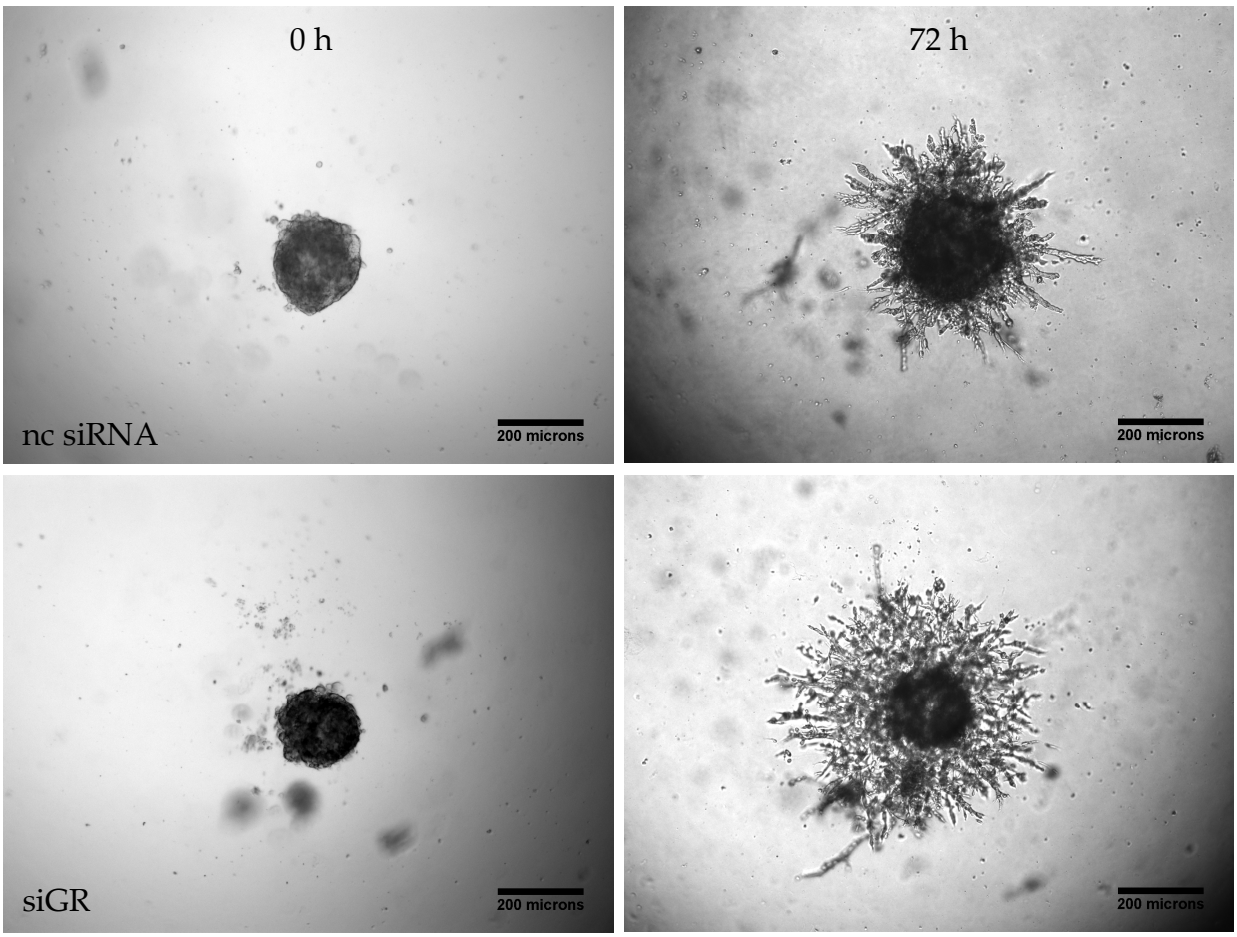

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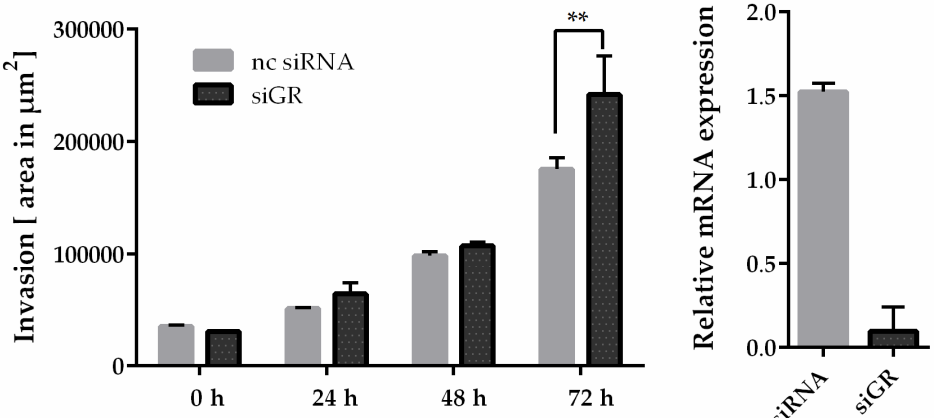

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Figure 4. Knockdown of the glucocorticoid receptor alpha (GRα) increased invasiveness of triple negative breast cancer. (**A**) Representative images of tumor cell invasion into a 3D matrix. Knockdown of GRα was performed with the Lonza Nucleofector 2b device on day one. On day three, $3*10^3$ cells per well were seeded into ultra-low-attachment (ULA) round bottom plates. Formation of spheroids was allowed for 72 hours. At t= 0, matrigel was added to the spheroids to start invasion. Scale bars in black show 200 microns in length. (**B**) The invasion is depicted over a time range of three days and the area of invaded cells into matrigel was calculated with imageJ FIJI at

the respective time points. (**C**) As confirmed by qPCR analysis, GRα expression is reduced up to 94% after 72 hours. The data represent means of three independent experiments. Error bars were calculated using ±SEM. P-values were calculated against nc siRNA control. Two stars represent a significance of $p<0.01$.

2.5. Pseudopterosin Inhibited Cytokine Release in a Co-Culture of Primary Blood Mononuclear Cells (PBMC) and Triple Negative Breast Cancer Cells

The microenvironment plays a critical role in breast cancer carcinogenesis[32]. Tumor associated macrophages are the drivers of intravasation of breast cancer cells[33,34], representing over 50 % of the tumor mass[5]. A main characteristic of inflammatory breast cancer is the secretion of pro-inflammatory cytokines such as IL-6 or IL-8 by macrophages, regulating angiogenesis and promoting tumor progression[35,36]. Previously, we verified a blockade of NF-κB-dependent cytokine expression and secretion after pseudopterosin treatment in both, MDA-MB-231 and THP-1 cells [16]. In this context, GRα knockdown led to the failure of pseudopterosin to inhibit cytokine expression. Furthermore, applying conditioned media, pseudopterosin revealed inhibitory activity in bidirectional communication between MDA-MB-231 and THP-1 cells. In the current work we verified a significant reduction of cytokine expression after PsA-D treatment in peripheral blood mononuclear cells (PBMC) (Supplemental Fig. S3). To further evaluate the pharmacological effects of pseudopterosin on bidirectional communication, we set up a co-culture encompassing PBMC and MDA-MB-231 cells to analyze the change in IL-6 and IL-8 expression levels. In the co-culture model, PsA-D treatment inhibited IL-6 expression significantly by 52.6% and IL-8 expression by 76.8%, respectively (Table 1). The fold increase of the IL-6 expression level in co-culture increased by 1.9 compared to mono-culture (Fig. 5). As expected, PsA-D treatment reduced IL-6 expression levels by 3.5 fold (Fig. 5). To further explore the agonism of pseudopterosin and GRα in the context of our co-culture model, the focus in our next study will lay in continuing investigations concerning knockdown studies of GRα. Taking together, our data indicate that pseudopterosin has the potential to inhibit the proliferation, the invasiveness and the communication of PBMC and MDA-MB-231 cells in a co-culture model. Thereby, the inhibitory activity of pseudopterosin seems to depend on GRα expression.

Table 1. Inhibition of cytokine expression in co-culture of peripheral blood mononuclear cells (PBMC) and MDA-MB-231 cells after pseudopterosin treatment.

PBMC	MDA-MB-231	Co-culture	Co-culture +PsA-D	P-value	% Inhibition[3]
1.09 (± 3.2) [1]	31.7 (± 20.3) [1]	44.6 (± 25.3) [1]	21.2 (± 12.7) [1]	0.02	52.6
27.1 (± 36.9) [2]	67.9 (± 46.5) [2]	213.9 (± 99.6) [2]	49.5 (± 13.2) [2]	0.22	76.8

[1] relative mRNA expression of IL-6

[2] relative mRNA expression of IL-8

[3] % inhibition was calculated as follow: 'co-culture' equates 100% (a), proportion of 'co-culture + PsA-D' to 'co-culture' (b); difference between (a) and (b).

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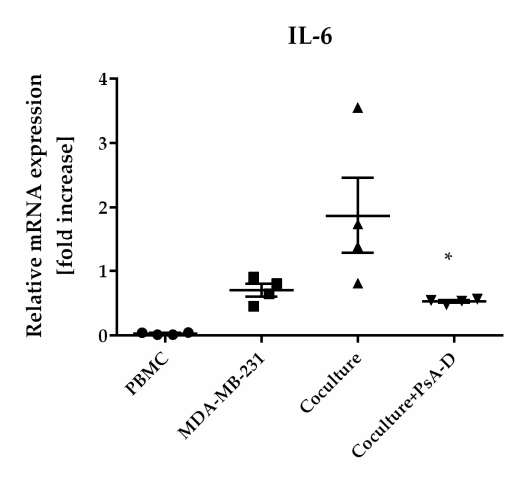

Figure 5. Pseudopterosin inhibited cytokine expression in a co-culture of PBMC and MDA-MB-231. Both cell lines were co-cultured at a ratio of 1:1 before treatment with 30 µM PsA-D. Cells were harvested 24 hours after treatment and cytokine expression levels were analyzed with qPCR. Data represent means of four independent experiments. Standard deviation was calculated using ±SEM. P-values were calculated between 'co-culture' and 'co-culture + PsA-D' using Dunnett's multiple comparisons test.

3. Discussion

For pseudopterosin effective biological activities in various therapeutic areas including anti-inflammatory effects are described[9–11]. This study aimed to explore the inhibitory capabilities of pseudopterosin on distinct features of triple negative breast cancer (TNBC), namely the ability to invade surrounding tissue and the contribution to rapid tumor progression. For TNBC, a disease with a high unmet medical need and a low survival rate, we demonstrated previously a novel potential of pseudopterosin by inhibiting NF-κB signaling and subsequent cytokine secretion[16]. Furthermore, proved by the translocation of GRα, we revealed a role of GRα activation upon pseudopterosin treatment. In the current study, GRα again proved to have an essential role in mediating pseudopterosin induced inhibition of breast cancer cell proliferation.

Among others, NF-κB is an important regulator in the development of the mammary glands[37]. However, chronic inflammation in general and inflammation in the tumor microenvironment in particular, caused by NF-κB up-regulation over a long time range, increases aggressiveness, invasivenes[38,39] and correlates with poor prognosis in breast cancer patients[40]. As we proved pseudopterosin to inhibit constitutive NF-κB activity in TNBC cells[16], we further examined effects of pseudopterosin on blocking invasion. Adipocytes in breast tumors are described to secrete high amounts of collagen leading to increased tumor growth[41]. Despite of using equivalently high collagen concentrations, which is known to reduce drug sensitivity[42], pseudopterosin displayed strong anti-invasive properties. Moreover, in a GRα knockdown, invasiveness in breast cancer tumor spheroids increased.

Gene expression analysis of breast tumors revealed a down-regulation of genes involved in cell differentiation, whereas genes promoting tumorigenesis were up-regulated[43]. However, mutations alone cannot explain the high malignancy and the complexity of the tumor. The tumor microenvironment is the most important factor of why immune cells undergo a reprogramming step, thereby promoting tumor progression. The discovery that normal mammary epithelial cells cooperate with innate immune cells for invasive processes, led to the discovery that macrophages are the drivers of intravasation from invasive breast tumors by establishing the tumor microenvironment[33,44]. Thereby, extracellular matrix (ECM), stromal cells such as endothelial and immune cells, fibroblasts and adipocytes are the main components of the microenvironment[45]. Additionally, tumor associated macrophages (TAMs) play a critical role in the tumor microenvironment by secreting second messengers such as IL-8 or IL-6 via NF-κB activation, thus

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promoting the tumor microenvironment and regulating angiogenesis which in turn correlates with poor outcome and malignant features in breast cancer[35,36,46,47]. Paradoxically, cytotoxic chemotherapy further initiates TAM recruitment into invasive carcinoma[48], where co-culture with breast cancer cells results in high IL-6 levels leading to activation of cancer stem cells[49]. We confirmed elevated IL-6 and IL-8 expression levels as a result of co-cultivating PBMC and MDA-MB-231 cells, where pseudopterosin was able to significantly block cytokine expression and henceforth the communication of both cell types.

In the clinics, glucocorticoids are used to reduce allergic reactions or nausea during chemotherapy due to up-regulation of anti-inflammatory signals [50–52]. On tumor cells, the synthetic GRα ligand dexamethasone (Dex) has been described to reduce cell proliferation by decreasing ERK phosphorylation in ER$^+$ breast cancer cells, possibly via the mechanism of transactivation[51]. ERK is a key regulator of proliferation and remodels the chromatin structure[29]. To our knowledge, anti-proliferative effects of Dex where as yet not observed in MDA-MB-231 cells. In contrast, Dex was described to increase tumor growth and act pro-proliferative[53]. However, in our study, we not only observed anti-proliferative effects after Dex treatment, but also witnessed improved anti-proliferative effects of pseudopterosin treatment compared to Dex. Interestingly, preliminary data indicate that the mechanism of action of pseudopterosin seems to be distinct from Dex, as the phosphorylation status of ERK did not change in the presence of pseudopterosin.

To date, GRα signaling can be divided into two distinct pathways: the so-called "transactivation", reflecting target gene expression, and the "transrepression", representing the downregulation of parallel signaling pathways such as NF-κB activation. Prominent metabolic side effects of glucocorticoid treatment might be ascribed to transactivation of GRα[54]. In contrast, positive effects of glucocorticoids include reduced migration and a reduction in proteins associated with chemotherapy resistance in TNBC cells, which might be explained by transrepression of GRα[55–57]. The mechanism of the transrepressive process of GRα can have different origins: GRα can heterodimerize and bind directly to the p65/p50 dimer[58] or GRα recruits histone deacetylases to the promotors of inflammatory genes[59]. GRα transrepression is thereby defined as a direct interaction with transcription factors, for example NF-κB, without binding to DNA response elements and independent of IκB, p50 or p65 regulation of expression[54]. Thus, up-regulation of IκBα expression[60] or repression of IL-8 by transcriptional inhibition of NF-κB are correlated with transactivation of GRα[54]. After knockdown of GRα, we observed increased invasiveness in tumor spheroids and a lack of pseudopterosin to inhibit proliferation or invasion. Thus, we suggest the expression of GRα to be beneficial in maintaining a less invasive phenotype in TNBC and propose pseudopterosin to address the mechanism of transrepression by agonizing GRα.

In conclusion, we demonstrated potent inhibitory effects of pseudopterosin on pronounced characteristics of TNBC including tumor cell proliferation and invasion. Our results imply pseudopterosin as a potential therapeutic basis suitable for targeting TNBC. Future studies will focus on investigating the molecular function including transrepressive effects of GRα in mediating pseudopterosin-dependent pharmacological actions.

4. Materials and Methods

4.1 Cell Culture and Reagents

The origin of the extract of pseudopterosin A to D isolated from *A. elisabethae* (subsequently named PsA-D) was kindly provided by Dr. Russell Kerr (University of Prince Edward Island, Marine Natural Products Lab, Canada) as described in our previous work[16]. U0126 inhibitor was purchased from Selleckchem (Houston, U.S.). MDA-MB-231 breast cancer cells were obtained from European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and grown in humidified atmosphere containing 5% CO_2 in RPMI medium. Medium was supplemented with 15 % FCS, 100 units ml^{-1} penicillin and 100 µg ml^{-1} units streptomycin. PBMCs were purchased from STEMCELL Technologies (Vancouver, Canada) and cultured in the presence of 5% CO_2 in RPMI

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along with 10% FCS, penicillin and streptomycin. Staurosporin was purchased from Sigma-Aldrich (St. Louis, USA) and medium and antibiotics from Life Technologies (Gibco, Carlsbad, U.S.).

4.2 Realtime Cell Proliferation

MDA-MB-231 breast cancer cells were seeded at a density of $1*10^5$ cells per ml in 96-well image lock plates (Sartorius, Goettingen, Germany) and images were taken every hour for a time frame of five days with the IncuCyte® Zoom from Sartorius (Goettingen, Germany). Confluency of cells was determined using the software of IncuCyte® Zoom (Version 2016B).

4.3 Knockdown Studies

Glucocorticoid receptor alpha (GRα) siRNA (siGRα) sc-35505 was purchased from Santa Cruz Biotechnology (Dallas, U.S.). Silencer® Select Negative Control No. 2 siRNA (nc siRNA) was obtained from Life Technologies (Carlsbad, U.S.). $1*10^6$ cells were transfected with 300 nM siRNA using the Nucleofector 2b device (Lonza, Basel, Switzerland) using the X-013 protocol for transfection of MDA-MB-231 cells. After different time points, cells were harvested and expression upon knockdown of interest was analyzed using quantitative realtime PCR, respectively.

4.4 Quantitative Realtime PCR

To determine cytokine or GRα expression levels after co-culture or knockdown, the following primers were used (purchased from Eurofins, Ebersberg, Germany): IL-6 forward (GGCACTGGCAGAAAACAACC), IL-6 reverse (GCAAGTCTCCTCATTGAATCC) IL-8 forward: (ACTGAGAGTGATTGAGAGTGGAC), IL-8 reverse: (AACCCTCTGCACCCAGTTTTC), GAPDH forward: (TGCACCACCAACTGCTTAGC), GAPDH reverse: (GGCATGGACTGTGGTCATGAG), GR forward: (AAAAGAGCAGTGGAAGGACAGCAC) GR reverse: (GGTAGGGGTGAGTTGTGGTAACG). Total RNA was isolated with "RNase Mini kit" from QIAGEN (Hilden, Germany) according to the manufacturer's instructions and reverse transcriptase PCR was performed using "Reverse Transcription Kit" from Promega (Darmstadt, Germany). Realtime PCR was conducted with "Quantitect SYBR Green" from QIAGEN based on the following protocol: Pre-incubation at 95° for 900 seconds, amplification was performed over 45 cycles (95° for 15 seconds, 55° for 25 seconds and 72° for 10 seconds). No-template controls served as negative controls. C_T values were calculated according to the $2^{-\Delta\Delta C_T}$ method[61]. Sample values were normalized to the house-keeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

4.5 3D Invasion Assay

To study MDA-MB-231 invasion into an extracellular matrix such as matrigel (Corning, New York, U.S.), spheroids of MDA-MB-231 were generated for 72 hours starting with $3*10^3$ cells and 0.25% matrigel in an ultra-low-attachment (ULA) plate (Corning, New York, U.S.). Invasion was initiated by addition of matrigel in a ratio of 1:1 volume to the spheroids. Images were taken with the IncuCyte® Zoom (Sartorius, Goettingen, Germany) to create a time lapse movie or the Axio Vert.A1 microscope (Zeiss, Oberkochen, Germany) every 24 hours for a time frame of three days. Image analysis was done with imageJ makro "Analyze Spheroid Cell Invasion in 3D matrix" by Volker Baecker[62] (FIJI distribution[63]).

4.6 Co-culture Studies

Co-culture of PBMC and MDA-MB-231 cells: PBMC were freshly thawed for each experiment. $1*10^6$ cells of MDA-MB-231 were seeded on day one and incubated with PsA-D for 20 minutes on day two. Treatment was followed by addition of PBMC cells to the MDA-MB-231 cells at a ratio of 1:1. Finally, cells were harvested at day three and analyzed for cytokine expression by realtime PCR.

4.7 Preparation of PsA-D Mixture

A. elisabethae was collected from South Bimini Island, as described I our previous work[16]: the extract was dried and extracted in EtOAc/MeOH (1:1) for 48 hours and subjected to silica gel chromatography eluting with hexanes and EtOAc to afford a mixture of PsA-D. The ratio was determined to be 85:5:5:5 (PsA:B:C:D) by LC-MS analysis.

4.8 Statistical Analysis

All data shown represent at least three independent experiments. Error bars show ±SEM of all the means of triplicate values. Figures and statistical analysis were generated with Graphpad Prism v. 6.07 (Graphpad Software, San Diego, USA) using one-way-ANOVA and the underlying Dunnett's multiple comparisons test. $P<0.05$ was chosen to define statistically significant differences.

Supplementary Material: Figure S1: Cell Viability of MDA-MB-231 cells after pseudopterosin treatment. Figure S2: Pseudopterosin failed to inhibit breast cancer cell proliferation after knockdown of the glucocorticoid receptor alpha (GRα) after 72 hours. Figure S3: Pseudopterosin inhibited bidirectional communication between triple negative breast cancer (TNBC) and peripheral blood mononuclear cells (PBMC).

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Author Contributions: Nicole Teusch and Julia Sperlich developed the scientific concept and designed the experiments. Julia Sperlich performed the experiments and analyzed the data. Nicole Teusch and Julia Sperlich wrote the manuscript.

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