# 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 $\alpha$ (GR$\alpha$) revealed that anti-proliferative effects of pseudopterosin were significantly inhibited when GR$\alpha$ expression was reduced. Furthermore, pseudopterosin inhibited invasion of MDA-MB-231 3D tumor spheroids embedded in an extracellular-like matrix. Remarkably, the knockdown of GR$\alpha$ 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 indicated 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) 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

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

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

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

63  **2. Results**

64  *2.1. Pseudopterosin Inhibited Proliferation of Triple Negative Breast Cancer Cells*

65      In our previous work we identified the natural product pseudoperosin as a novel inhibitor of
66  NF-κB signaling[16], one key pathway in controlling progression of TNBC. As NF-κB is known to
67  regulate various processes in cancer progression such as proliferation, angiogenesis or invasion[26–28],
68  the aim of the current study was to further characterize the pharmacological properties of
69  pseudopterosin. First, we investigated a pseudopterosin extract (PsA-D) regarding its effect on
70  breast cancer cell proliferation in MDA-MB-231 cells. To remain within a non-toxic concentration
71  range of PsA-D (IC$_{50}$ values of cell viability for PsA-D after 24 hours or 48 hours of treatment were
72  31.4 μM and 32.2 μM, respectively; Supplemental Fig. S1A/B), 7.5 and 15 μM of PsA-D were chosen
73  to evaluate anti-proliferative effects (Fig. 1A). As expected, MDA-MB-231 cells treated with DMSO
74  showed a high proliferation rate, represented by a confluency of 78% after 48 hours (Fig. 1A).
75  Notably, a concentration of 15 μM of PsA-D was able to reduce proliferation significantly after 24
76  hours by 1.9 fold and after 48 hours by 1.6 fold compared to DMSO control (Fig. 1B and 1C).
77  Furthermore, preliminary data indicate that pseudopterosin-induced reduction of proliferation is
78  not pERK dependent (Supplemental Fig. S3), which is a key regulator for cell proliferation in
79  principle[29].

(A)

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(B)                    (C)

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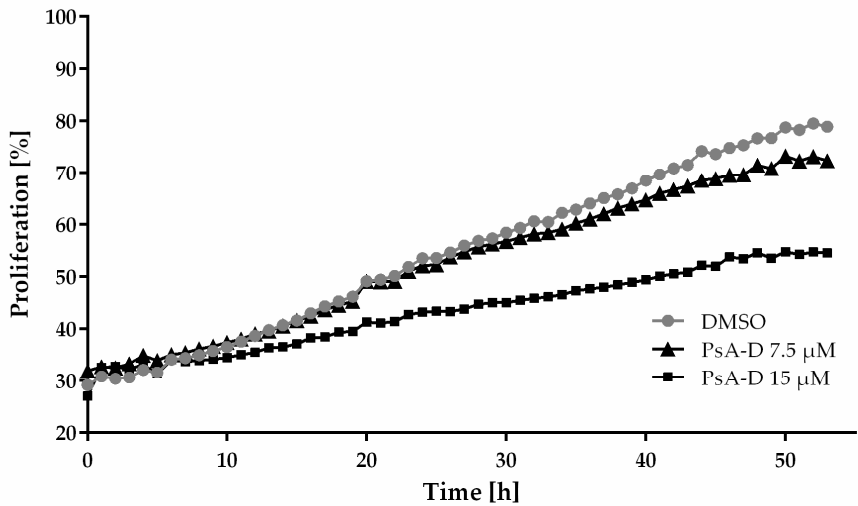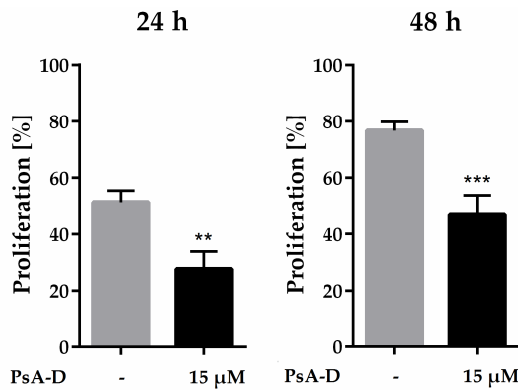
82   **Figure 1. Pseudopterosin inhibited proliferation in triple negative breast cancer cells.** (**A**)
83   Proliferating cells were imaged every hour over a time range of 50 hours with the IncuCyte® ZOOM.
84   Confluency of cells was determined with IncuCyte® software indicated as proliferation in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

90   *2.2. Glucocorticoid Receptor Alpha Expression is Essential for Anti-Proliferative Effects of Pseudopterosin*

91   In our previous work we hypothesized pseudopterosin to act as an agonist of the glucocorticoid
92   receptor alpha (GRα)[16]. Subsequently, when downregulating GRα, pseudopterosin failed to inhibit
93   NF-κB target gene expression. Thus, to further explore the role of GRα in the mode-of-action of
94   pseudopterosin, we analyzed the effect of a GRα knockdown on breast cancer cell proliferation.
95   After 72 and 85 hours, treatment with PsA-D inhibited proliferation in non-coding siRNA (nc
96   siRNA) transfected cells by 2 fold, respectively (Fig. 2A and Supplementary Fig. S4). Importantly, in
97   siGRα transfected cells, PsA-D lost its anti-proliferative effect (Fig. 2A). Efficiency of the GRα
98   knockdown using realtime qPCR (up to 88%) is exemplified in Fig. 2B and depicted on the protein
99   level via immunfluorescence analysis in Fig. 2C. In conclusion, our data suggests that GRα
100  expression might be crucial for the anti-proliferative effects of PsA-D.
101  Notably, treatment with the marked GRα ligand dexamethasone showed less potency in
102  reducing proliferation: after 48 hours, PsA-D resulted in a 21% proliferation decrease, whereas 100

103    nM dexamethasone reduced proliferation by 15% compared to DMSO, respectively (Fig. 2C). After
104    72 hours, PsA-D treatment diminished proliferation by 20%, whereas treatment with 100 nM
105    dexamethasone reduced the proliferation rate by only 9% (Fig. 2D).

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(D)                                                           (E)
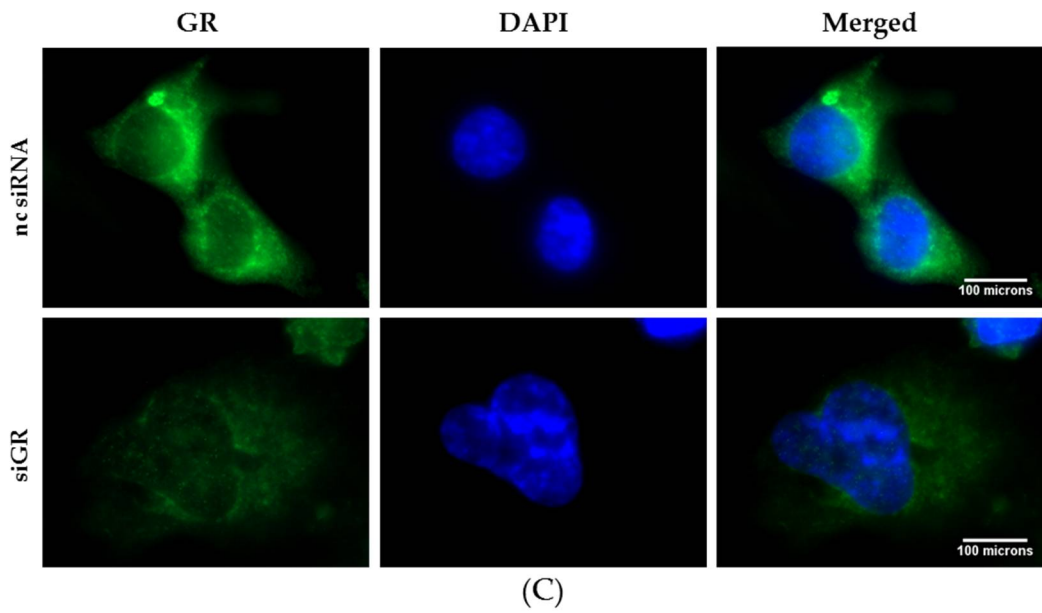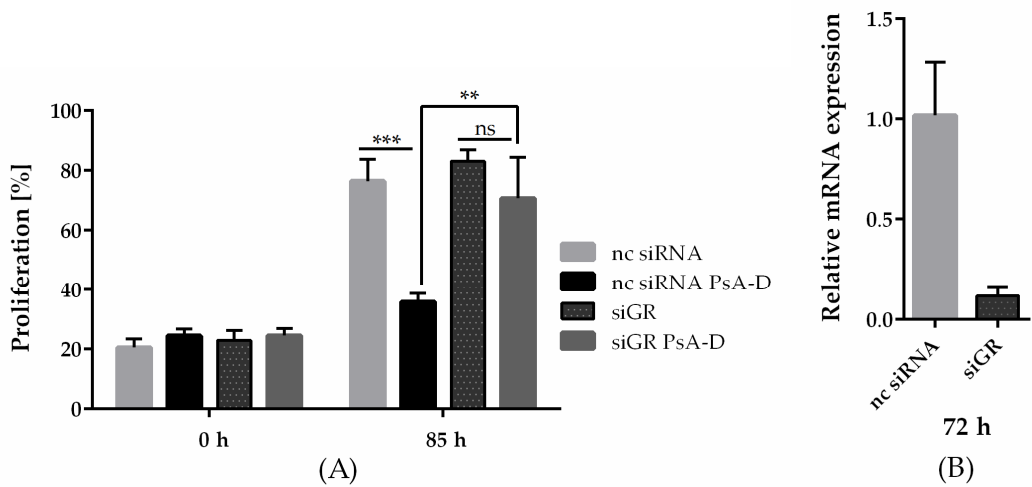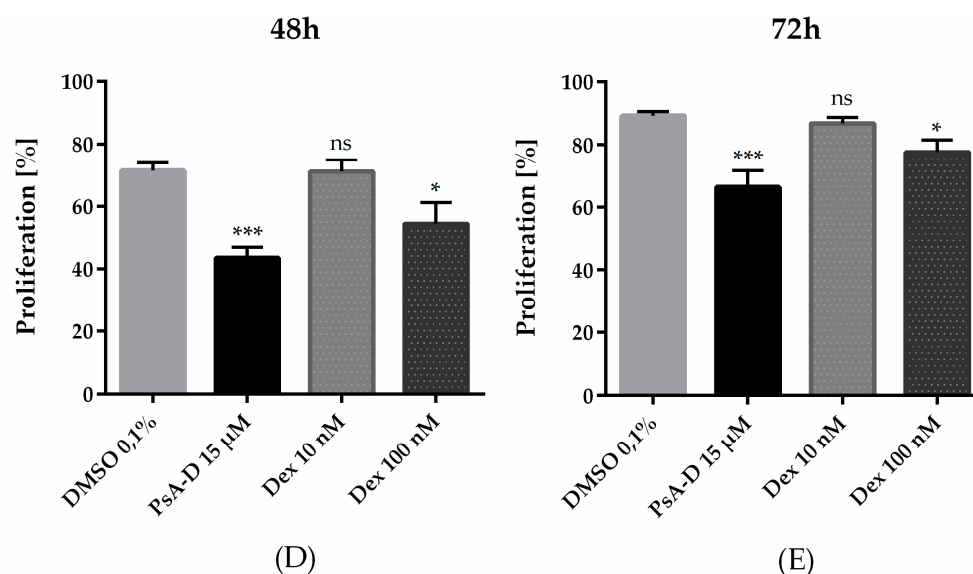
**Figure 2. Pseudopterosin failed to inhibit breast cancer cell proliferation after knockdown of the glucocorticoid receptor alpha (GRα) and inhibited proliferation of MDA-MB-231 more efficaciously than dexamethasone (Dex).** (**A**) Knockdown of GRα was done with the Lonza Nucleofector 2b device on 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**) Immunofluorescent analysis of GRα knockdown after 72 hours. Scale bars in white show 100 microns in length. (**D-E**) 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 harbor many devastating characteristics 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 effects of pseudopterosin on the 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 significantly inhibited the invasion of single cells into the matrix. 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). Thus, in our 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.

(A)

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(B)        (C)        (D)

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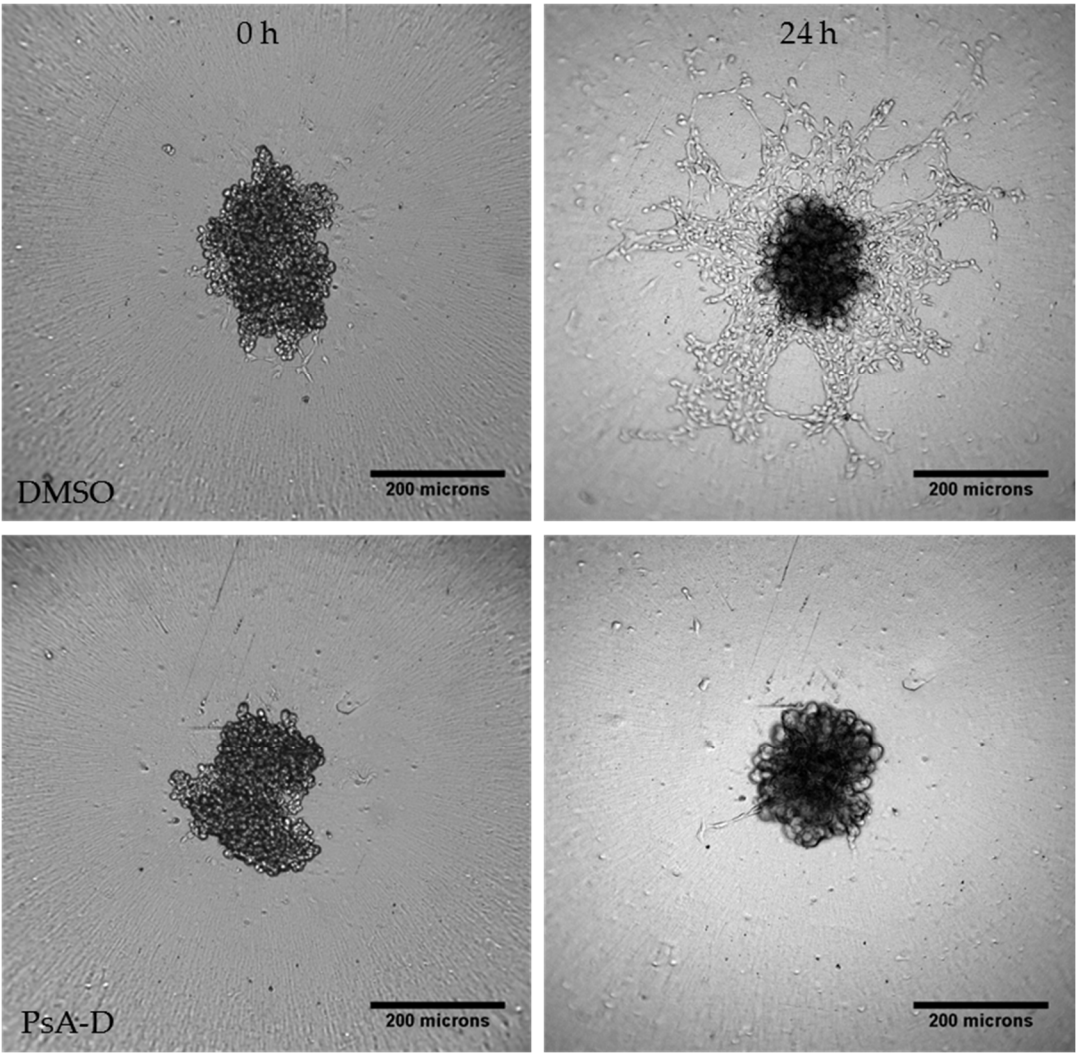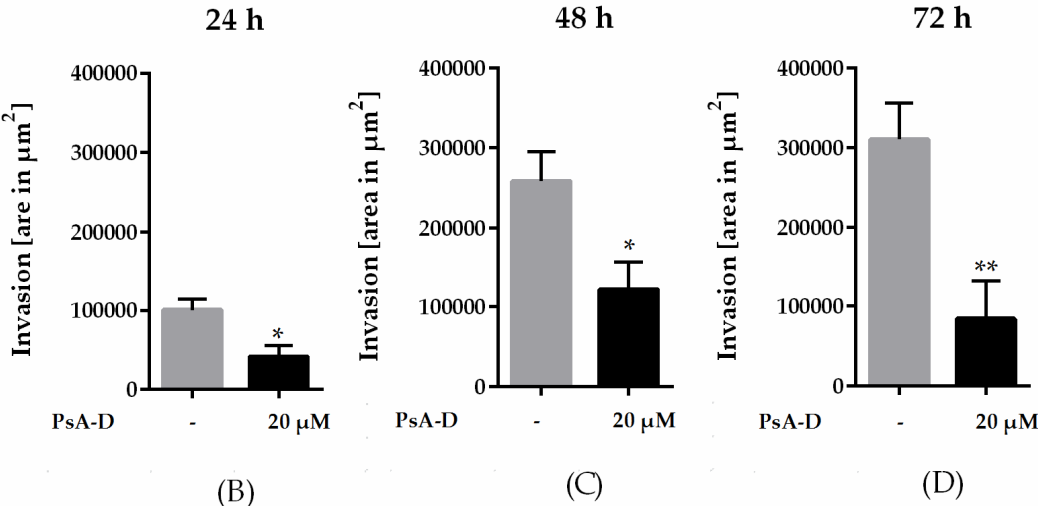
136   **Figure 3. Pseudopterosin inhibited invasion into a 3D matrix.** (**A**) Representative images of
137   invasion of cells into a 3D matrix at 24 hours' time point. Cells were imaged with IncuCyte® ZOOM
138   over a time range of three days. $3*10^3$ cells per well were seeded into ULA round bottom plates and
139   spheroids were formed for 72 hours. Scale bars in black show 200 microns in length. (**B-D**) The bar
140   diagrams show three different time points representing six independent experiments. Spheroids
141   were treated with a concentration of 20 μM of PsA-D. Error bars were calculated using ±SEM.

142    P-values were calculated against control (CTRL). Two stars represent a significance of p<0.01 and one
143    star represents a significance of p<0.05.

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

145    The clinical use of glucocorticoids (GC) is discussed controversially, due to extensive side
146    effects, chemotherapy resistance and survival of cancer cells[21,23,31]. However, recent literature
147    indicates the beneficial effects of GCs to be strongly dependent on the tumor entity: survival in
148    patients receiving GC combined with anthracycline-based chemotherapy was improved[25]. In this
149    context, we further investigated the role of GRα in the invasiveness of MDA-MB-231 micro-tumor
150    spheroids (Fig. 4A). The efficiency in GRα knockdown is represented by a reduction of 94% (Fig. 4C).
151    After 72 hours, the spheroids transfected with siGRα showed a significant increase in invasion by
152    27% compared to nc siRNA (Fig. 3B). In conclusion, the knockdown of GRα led to an elevation of
153    invasiveness in MDA-MB-231 cells, suggesting a potential of GRα agonists like pseudopterosin in
154    diminishing TNBC progression.

(A)

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(B)                                                                 (C)

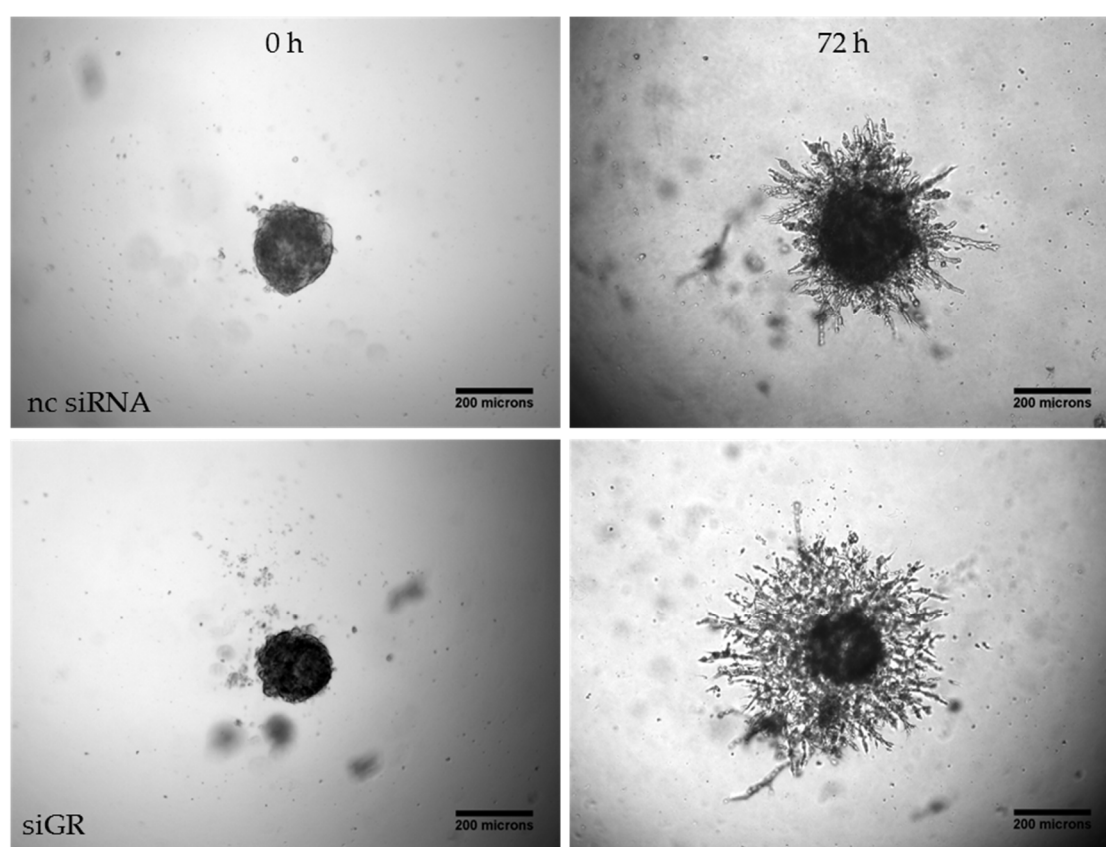
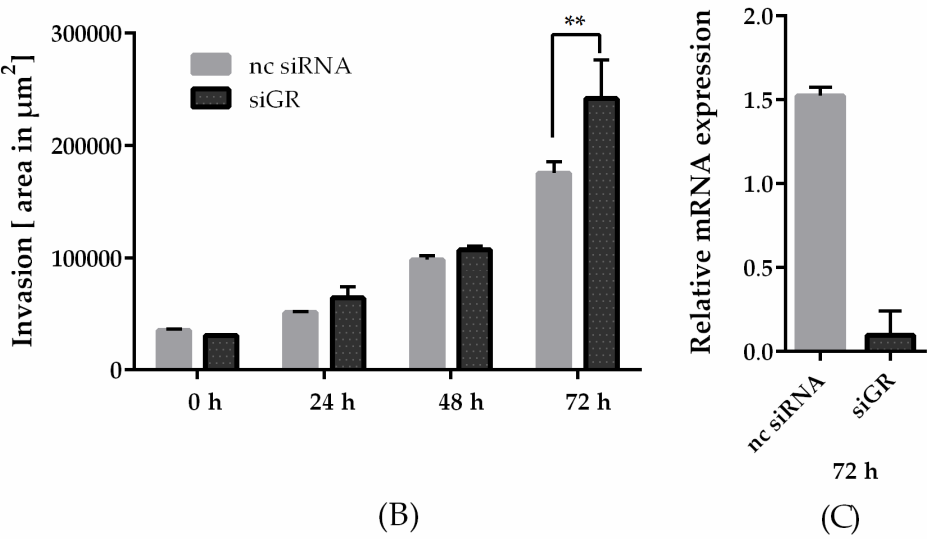



**Figure 4. Knockdown of the glucocorticoid receptor alpha (GRα) increased invasiveness of triple negative breast cancer.** (**A**) Representative images of tumor cell invasion into a 3D matrix. Knockdown of GRα was performed with the Lonza Nucleofector 2b device on day one. On day three, $3*10^3$ cells 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.

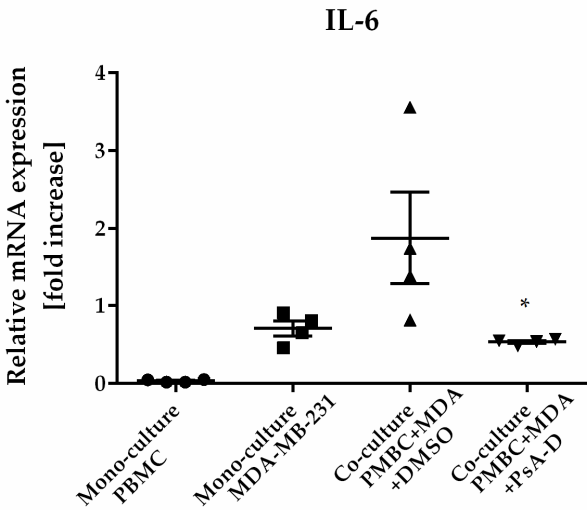
*2.5. Pseudopterosin Inhibited Cytokine Release in a Co-Culture of Primary Blood Mononuclear Cells (PBMC) and Triple Negative Breast Cancer Cells*

The microenvironment plays a critical role in breast cancer carcinogenesis[32]. Tumor associated macrophages are the drivers of breast cancer cells invasion[33,34]. 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, as shown previously, stimulation by the TLR4 ligand LPS leads to the production of cytokines and the subsequent secretion into the surrounding "conditioned medium" (CM) [16]. Our current data amend a significant reduction of cytokine expression, such as IL-6, IL-8 and TNFα, after PsA-D treatment in peripheral blood mononuclear cells (PBMC) (Supplemental Fig. S5). Medium containing cytokines released by MDA-MB-231 cells, representing the so called "MDA-MB-231 conditioned medium" (M-CM), induced a significant cytokine expression in PBMC. Notably, pseudopterosin treatment was able to block cytokine expression induced by breast cancer cell conditioned media in PBMC. (Supplemental Fig. S5). Thus, 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

188   increase of the IL-6 expression level in co-culture increased by 1.9 compared to mono-culture (Fig. 5).
189   As expected, PsA-D treatment reduced IL-6 expression levels by 3.5 fold (Fig. 5). To further explore
190   the agonism of pseudopterosin and GR$\alpha$ in the context of our co-culture model, the focus in future
191   studies will lay in continuing investigations concerning knockdown studies of GR$\alpha$. Taking
192   together, our data indicate that pseudopterosin has the potential to inhibit the proliferation, the
193   invasiveness and the communication of PBMC and MDA-MB-231 cells in a co-culture model.
194   Thereby, the inhibitory activity of pseudopterosin seems to depend on GR$\alpha$ expression.

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

| | Mono-culture PBMC | Mono-culture MDA* | Co-culture PBMC+MDA +DMSO | Co-culture PBMC+MDA +PsA-D | P-value[1] |
|---|---|---|---|---|---|
| IL-6[2] | 1.09 (± 3.2) | 31.7 (± 20.3) | 44.6 (± 25.3) | 21.2 (± 12.7) | 0.02 |
| IL-8[2] | 27.1 (± 36.9) | 67.9 (± 46.5) | 213.9 (± 99.6) | 49.5 (± 13.2) | 0.22 |

197   [1] P-values were calculated with ONE-Way ANOVA between 'co-culture' and 'co-culture + PsA-D'.
198   [2] The data represent relative mRNA expression values measured with realtime qPCR; * MDA is
199   equivalent for MDA-MB-231 cells.

201   **Figure 5. Pseudopterosin inhibited cytokine expression in a co-culture of PBMC and**
202   **MDA-MB-231.** Both cell lines were co-cultured at a ratio of 1:1 before treatment with 30 μM PsA-D.
203   Cells were harvested 24 hours after treatment and cytokine expression levels were analyzed with
204   qPCR. Relative mRNA levels were normalized to fold increase. MDA is equivalent for MDA-MB-231
205   cells. Data represent means of four independent experiments. Standard deviation was calculated
206   using ±SEM. P-values were calculated between 'co-culture' and 'co-culture + PsA-D' using Dunnett's
207   multiple comparisons test.

## 3. Discussion

209   For pseudopterosin effective biological activities in various therapeutic areas including
210   anti-inflammatory effects are described[9–11]. This study aimed to explore the inhibitory capabilities of
211   pseudopterosin on distinct features of triple negative breast cancer (TNBC), namely the ability to
212   invade surrounding tissue and the contribution to rapid tumor progression. For TNBC, a disease
213   with a high unmet medical need and a low survival rate, we demonstrated previously a novel
214   potential of pseudopterosin by inhibiting NF-κB signaling and subsequent cytokine secretion[16].

215  Furthermore, suggested by the translocation of GRα, we revealed a role of GRα activation upon
216  pseudopterosin treatment. In the current study, GRα again indicated to play a role in mediating
217  pseudopterosin induced inhibition of breast cancer cell proliferation.

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

228      Gene expression analysis of breast tumors revealed a down-regulation of genes involved in cell
229  differentiation, whereas genes promoting tumorigenesis were up-regulated[43]. However, mutations
230  alone cannot explain the high malignancy and the complexity of the tumor. The tumor
231  microenvironment is the most important factor of why immune cells undergo a reprogramming
232  step, thereby promoting tumor progression. The discovery that normal mammary epithelial cells
233  cooperate with innate immune cells for invasive processes, led to the discovery that macrophages
234  are the drivers of intravasation from invasive breast tumors by establishing the tumor
235  microenvironment[33,44]. Thereby, extracellular matrix (ECM), stromal cells such as endothelial and
236  immune cells, fibroblasts and adipocytes are the main components of the microenvironment[45].
237  Additionally, tumor associated macrophages (TAMs) play a critical role in the tumor
238  microenvironment by secreting second messengers such as IL-8 or IL-6 via NF-κB activation, thus
239  promoting the tumor microenvironment and regulating angiogenesis which in turn correlates with
240  poor outcome and malignant features in breast cancer[35,36,46,47]. Paradoxically, cytotoxic chemotherapy
241  further initiates TAM recruitment into invasive carcinoma[48], where co-culture with breast cancer
242  cells results in high IL-6 levels leading to activation of cancer stem cells[49]. We confirmed elevated
243  IL-6 and IL-8 expression levels as a result of co-cultivating PBMC and MDA-MB-231 cells, where
244  pseudopterosin was able to significantly block cytokine expression and henceforth the
245  communication of both cell types.

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

257      To date, GRα signaling can be divided into two distinct pathways: the so-called
258  "transactivation", reflecting target gene expression, and the "transrepression", representing the
259  downregulation of parallel signaling pathways such as NF-κB activation. Prominent metabolic side
260  effects of glucocorticoid treatment might be ascribed to transactivation of GRα[54]. In contrast, positive
261  effects of glucocorticoids include reduced migration and a reduction in proteins associated with
262  chemotherapy resistance in TNBC cells, which might be explained by transrepression of GRα[55–57].
263  The mechanism of the transrepressive process of GRα can have different origins: GRα can
264  heterodimerize and bind directly to the p65/p50 dimer[58] or GRα recruits histone deacetylases to the
265  promotors of inflammatory genes[59]. GRα transrepression is thereby defined as a direct interaction
266  with transcription factors, for example NF-κB, without binding to DNA response elements and

267  independent of IκB, p50 or p65 regulation of expression[54]. Thus, up-regulation of IκBα expression[60]
268  or repression of IL-8 by transcriptional inhibition of NF-κB are correlated with transactivation of
269  GRα[54]. After GRα knockdown, we observed increased invasiveness in tumor spheroids and a lack of
270  pseudopterosin to inhibit proliferation or invasion. Thus, we suggest the expression of GRα to be
271  beneficial in maintaining a less invasive phenotype in TNBC and propose pseudopterosin to address
272  the mechanism of transrepression by agonizing GRα.

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

278  **4. Materials and Methods**

279  *4.1. Cell Culture and Reagents*

280       The origin of the extract of pseudopterosin A to D isolated from *A. elisabethae* (subsequently
281  named PsA-D) was kindly provided by Dr. Russell Kerr (University of Prince Edward Island,
282  Marine Natural Products Lab, Canada) as described in our previous work[16]. U0126 inhibitor was
283  purchased from Selleckchem (Houston, U.S.). MDA-MB-231 breast cancer cells were obtained from
284  European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and grown in
285  humidified atmosphere containing 5% $CO_2$ in RPMI medium. Medium was supplemented with 15 %
286  FCS, 100 units $ml^{-1}$ penicillin and 100 µg $ml^{-1}$ units streptomycin. PBMCs were purchased from
287  STEMCELL Technologies (Vancouver, Canada) and cultured in the presence of 5% $CO_2$ in RPMI
288  along with 10% FCS, penicillin and streptomycin. Staurosporin was purchased from Sigma-Aldrich
289  (St. Louis, USA) and medium and antibiotics from Life Technologies (Gibco, Carlsbad, U.S.).

290  *4.2. Realtime Cell Proliferation*

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

295  *4.3. Knockdown Studies*

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

302  *4.4. Quantitative Realtime PCR*

303       To determine cytokine or GRα expression levels after co-culture or knockdown, the following
304  primers were used (purchased from Eurofins, Ebersberg, Germany): IL-6 forward
305  (GGCACTGGCAGAAAACAACC), IL-6 reverse (GCAAGTCTCCTCATTGAATCC) IL-8 forward:
306  (ACTGAGAGTGATTGAGAGTGGAC), IL-8 reverse: (AACCCTCTGCACCCAGTTTTC), GAPDH
307  forward: (TGCACCACCAACTGCTTAGC), GAPDH reverse: (GGCATGGACTGTGGTCATGAG),
308  GR          forward:          (AAAAGAGCAGTGGAAGGACAGCAC)          GR          reverse:
309  (GGTAGGGGTGAGTTGTGGTAACG). Total RNA was isolated with "RNase Mini kit" from
310  QIAGEN (Hilden, Germany) according to the manufacturer's instructions and reverse transcriptase
311  PCR was performed using "Reverse Transcription Kit" from Promega (Darmstadt, Germany).
312  Realtime PCR was conducted with "Quantitect SYBR Green" from QIAGEN based on the following

313  protocol: Pre-incubation at 95° for 900 seconds, amplification was performed over 45 cycles (95° for
314  15 seconds, 55° for 25 seconds and 72° for 10 seconds). No-template controls served as negative
315  controls. $C_T$ values were calculated according to the $2^{-\Delta\Delta C_T}$ method[61]. Sample values were normalized
316  to the house-keeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

*4.5. 3D Invasion Assay*

318  To study MDA-MB-231 invasion into an extracellular matrix such as matrigel (Corning, New
319  York, U.S.), spheroids of MDA-MB-231 were generated for 72 hours starting with $3*10^3$ cells and
320  0.25% matrigel in an ultra-low-attachment (ULA) plate (Corning, New York, U.S.). Invasion was
321  initiated by addition of matrigel in a ratio of 1:1 volume to the spheroids. Images were taken with the
322  IncuCyte® Zoom (Sartorius, Goettingen, Germany) to create a time lapse movie or the Axio Vert.A1
323  microscope (Zeiss, Oberkochen, Germany) every 24 hours for a time frame of three days. Image
324  analysis was done with imageJ makro "Analyze Spheroid Cell Invasion in 3D matrix" by Volker
325  Baecker[62] (FIJI distribution[63]).

*4.6. Co-culture Studies*

327  Co-culture of PBMC and MDA-MB-231 cells: PBMC were freshly thawed for each experiment.
328  $1*10^6$ cells of MDA-MB-231 were seeded on day one and incubated with PsA-D for 20 minutes on
329  day two. Treatment was followed by addition of PBMC cells to the MDA-MB-231 cells at a ratio of
330  1:1. Finally, cells were harvested at day three and analyzed for cytokine expression by realtime PCR.

*4.7. Preparation of PsA-D Mixture*

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

*4.8. Immunofluorescent staining*

337  After treatment according to *4.3*, cells were fixed with −10 °C cold methanol for 5 minutes and
338  treated with 0.1% Triton™ X-100 for 15 minutes. Antibodies were purchased from Santa Cruz
339  Biotechnology (Dallas, Texas, USA): primary antibody (sc-8992 GR (H-300)) incubated 1:50 for 24
340  hours overnight at 4°C and secondary antibody (sc-2012 IgG-FITC (fluorescein isothiocyanate)) was
341  incubated 1:100 for 2.5 hours at room temperature For staining the cell nuclei
342  4',6-Diamidin-2-phenylindol (DAPI, Sigma Aldrich, St. Louis, USA) was incubated for 5 min at room
343  temperature at a concentration of 3 µM. . Cells were washed three times with PBS following each
344  incubation step.

*4.9. Statistical Analysis*

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

350  **Supplementary Material:** Figure S1: Cell Viability of MDA-MB-231 cells after pseudopterosin
351  treatment. Figure S2: Cell viability assessment of PBMC cells after pseudopterosin treatment. Figure
352  S3: Pseudopterosin did not change ERK phosphorylation status in MDA-MB-231 cells. Figure S4:
353  Pseudopterosin failed to inhibit breast cancer cell proliferation after knockdown of the
354  glucocorticoid receptor alpha (GRα) after 72 hours. Figure S5: Pseudopterosin inhibited bidirectional
355  communication between triple negative breast cancer (TNBC) and peripheral blood mononuclear
356  cells (PBMC).

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

367  **Conflicts of Interest:** The authors declare no conflict of interest.

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