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## Violacein inhibits matrix metalloproteinase mediated CXCR4 expression: Potential anti-tumor effect in cancer invasion and metastasis

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### Abstract

Matrix metalloproteinases (MMP-2 and -9) play an important role in the tumor metastasis through cleavage of proinflammatory cytokines. Violacein a small molecule produced by *Chromobacterium violaceum* and has been implicated with anti-cancer effects. In this study we investigated the molecular basis of violacein mediated downregulation of CXCL12/CXCR4, chemokine-receptor ligand interaction. Zymography analysis demonstrated that violacein significantly inhibited the cytokine (TNF $\alpha$  and TGF $\beta$ ) mediated MMP-2 activation in MCF-7 breast cancer cell line. MMP-2 plays a critical role in the secretion of inflammatory chemokine, CXCL12, involved in cell migration and cancer metastasis. ELISA analysis demonstrated that violacein inhibited the secretion of CXCL12 from the activated MCF-7 cells. Further, we show that MMP-2/-9 act synergistically at two distinct steps towards the membrane expression of the tumor metastasis chemokine receptor, CXCR4. Violacein efficiently downregulated the CXCR4 membrane expression through MMP-9 inhibition. Taken together, these studies demonstrate a unique anti-tumor mechanism of action of violacein through reduction of CXCL12/CXCR4 interaction. These studies could offer a novel venue for violacein in cancer therapy.

### Keywords

violacein; cancer; cytokines; chemokines; matrix metalloproteinases; metastasis

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## 1. Introduction

Violacein a purple-colored indole derivative pigment produced by some of the *Chromobacterium violaceum* strains [1]. Violacein has been suggested to exert various biological properties including anti-cancer, anti-inflammatory, anti-bacterial, and anti-parasitic activities [2]. The complex ring structure of violacein renders it hydrophilic and thus with a potential to pass through the cell membrane with greater efficiency. Violacein due to its strong peak absorbance in the ultraviolet (UV) range it is suggested to be protective against UV irradiation damage [3]. The anti-tumor effects of violacein have been attributed in the leukemia cells cytotoxicity (at 1 mM concentration) through upregulation of apoptotic cascade proteins [4, 5]. Further, violacein has not been considered cytotoxic on normal healthy cells [4]. However, there is limited literature evidence on the effect of violacein on solid tumor models. In addition, antiparasitic role of violacein effecting various growth stages in the life-cycle of the parasites, *Trypanosoma* [6], *Leishmania* [7] and *Plasmodium* [8] have been shown. Along with this, anti-inflammatory and antipyretic effects of violacein have been demonstrated in in rat edema models [9]. However a precise molecular basis of these effects is still not clear. In our current studies we focused on the specific effect of violacein on migration or metastasis using established breast adenocarcinoma cell line, MCF-7.

In solid tumors, the metastatic ability of neoplastic cells to penetrate the basement membrane and initiate the inflammation mediated metastatic process is largely mediated by proteolysis [10]. Matrix metalloproteinases (MMPs) are particularly capable of degrading extracellular matrix (ECM) components [11]. These proteolytic enzymes are involved in connective tissue remodeling such as wound healing [12]. Moreover, abnormal production of these proteinases has been implicated in a number of cancers [11]. In this regard, chemokines, a super family of inflammatory proteins which are cleaved by MMPs have been attributed to induce cytoskeletal rearrangement and induce the cellular adhesion of neoplastic cells as well as their migration [13]. It has been well documented in literature that upregulation cellular inflammatory adhesion through chemokine-ligand, CXCL12–CXCR4, interactions play a critical role in the metastasis of breast cancer to lungs and lymph nodes [14]. In our current study, under *in vitro* conditions, we have tested the hypothesis that violacein inhibits the proteolytic MMPs and eventual down-regulation of CXCL12–CXCR4 interaction with for potential anti-cancer migration and invasion.

## 2. Materials and Methods

### 2.1. Cell culture

The human breast adenocarcinoma cell lines, MCF-7, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI-1640 culture medium at 37 °C in 5% CO<sub>2</sub> incubator until they were 80% confluent along with the supplements as suggested by the provider. Cell lines were frozen in liquid vapor nitrogen at –130°C until use. Upon thawing, cells were maintained in 5% CO<sub>2</sub> incubator in sterile essential media at 37°C. For cell stimulation studies, the cells were stimulated with TNF $\alpha$  (50 ng/mL) and TGF $\beta$  (50 ng/mL) with varying concentration of violacein (0-10  $\mu$ M) in DMSO for 72 hours. MCF-7 cells with only DMSO treatment were utilized as negative

controls in our study. siRNA (Santa Cruz Biotech, Dallas, TX) mediated gene knock-down of MMP-2 (sc-29398), MMP-9 (sc-29400) and CXCL12 antibody based neutralization (sc-133989) were performed for specific in vitro analysis along with negative controls (scramble siRNA and isotype control antibody). All other salts and chemicals (unless specifically mentioned) were obtained either from Sigma-Aldrich (St Louis, MO) and Life Technologies (Carlsbad, CA).

## 2.2. Violacein extraction

Violacein was purified from the *Chromobacterium violaceum* strain CV14N23, which was isolated from environmental soil and water samples collected in the Copper Basin of Tennessee. The bacterial cultures were grown for 48 hours in LB broth at 30°C with shaking to allow for pigment production. The violacein pigment was extracted from the cells and medium by adding ethanol to yield a 50% solution, and the solution was then centrifuged at 10,000 RPM to remove cells. The supernatant containing dissolved violacein was extracted with chloroform, and acid-base extractions to yield a purified (>90%) product based on RP-HPLC, UV-VIS spectrum and LCMS analyses.

## 2.3. MMP 2 and MMP9 activity determination

**MMP Zymogram**—The MMP2 and MMP9 activity in the cell cultures was determined by gelatin zymography (Invitrogen, Carlsbad, CA) as previously shown [15]. The gelatinolytic activity of the MMPs, were quantitatively analyzed by the optical density of the bands using the Bio-Rad Universal Hood II (Hercules, CA). Morphometric analysis was done using the software provided by the company.

**MMP activity**—The MMP2 and MMP9 enzymatic activity was further quantitated using gelatinase substrate activity as previously described [16] using MMP-2(RPN2631), MMP-9 (RPN2634), biotrack activity assay kit (GE Healthcare, Pittsburgh, PA) as per manufacturer protocols. The concentration of active MMP was interpolated from a standard curve obtained using the manufacturer provided standard.

## 2.4. MTT assay

Cell viability was measured by trypan blue dye exclusion (Sigma Aldrich, MO) and MTT assay (Life technologies, CA) as previously described [17]. Briefly, the effect of violacein and cytokines on viability of MCF-7 cells was assessed by measuring mitochondrial activity using MTT (4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay. The assay was performed as per manufacturer provided instructions and plates were read at 562 nm. Viability was calculated as percentage compared to untreated cells in basal conditions.

## 2.5. CXCL12 ELISA

The secretory extracellular CXCL12 in the MCF-7 cell supernatant was quantitated by ELISA. The CXCL12 ELISA was performed as per the manufacturer's protocol (R&D systems Inc, MN). The protein supernatant was diluted 1:1000 and quantified with a standard curve using the manufacturer provided standards. Detection at 450 nm was

performed using EMax Plus spectrophotometer and data analysis was carried out using software provided by the manufacturer (Molecular Devices, Sunnyvale, CA).

## 2.6. CXCR4 Flow cytometry

CXCR4 expression was analyzed by flow cytometry. The CXCR4 was labeled by mouse anti-CXCR4 primary antibody (Santa Cruz Biotech, TX) in 1:20 dilution to a 200  $\mu$ L final volume of MCF-7 cells ( $1 \times 10^6$  cells/mL). Antibodies used for flow cytometry included anti-mouse-FITC (BD Biosciences, San Jose, CA), and the samples were latter analyzed using a FACS Calibur/LSRII flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). Data were analyzed using BD FACSDiva software. Gates were set according to isotype controls.

## 2.7. Statistical analysis

Data are expressed as mean  $\pm$  SEM from four independent studies. Statistical differences between means were analyzed using a paired or unpaired Student's t test. A *p-value* of less than 0.05 was considered significant. All data analysis was obtained using Origin 7 software (Origin Labs, Northampton, MA) or GraphPad5 (Graph Pad Software, LaJolla, CA).

## 3. Results

### 3.1. Violacein inhibits MMP-2 activity

Pro-inflammatory mediated upregulation of MMPs have been demonstrated to play an important role in cancer metastasis [11]. To determine the anti-inflammatory and anti-tumor effect of violacein, we treated the activated (by TNF- $\alpha$  and TGF- $\beta$ , 50 ng/mL each) MCF-7 breast cancer cell line with 1  $\mu$ M violacein in DMSO. As shown in figure 1, activated MCF-7 induced 4 fold specific upregulation of MMP-2 (both pro-form and active-form) with no effect on MMP-9. Following treatment with violacein there was down modulation of MMP-2 expression from  $156 \pm 22$  to  $42 \pm 10$  (densitometric units),  $p < 0.05$ . However, vehicle DMSO alone treated activated MCF-7 cells did not demonstrate any reduction in MMP-2 expression, thus suggesting that the MMP-2 down modulation was mediated specifically by violacein. Recent literature evidence on MMPs have indicated that gelatin zymography studies only yield levels of MMP expression and not activity. Therefore we have performed additional MMP-2 specific gelatinase substrate activity assay. As shown in figure 1C, violacein inhibited the MMP-2 activity in activated MCF-7 cells from  $44 \pm 6$  U/ $\mu$ g to  $9 \pm 2$  U/ $\mu$ g,  $p < 0.05$ , with no effect on MMP-9 activity further confirming our zymography data. Further, dose dependent studies (figure 1E) have demonstrated that at 1  $\mu$ M concentration violacein was not cytotoxic. In our preliminary studies we noticed that the 50% cytotoxic effect of violacein was  $0.53 \pm 0.08$  mM concentration (data not shown) which is almost 500 fold higher than the concentration used in our current studies. Therefore, the current modulations in MMP-2 activity could be directly attributed to anti-inflammatory effect of violacein and not due to reduced cell viability.

### 3.2. Violacein inhibits MMP-2 mediated CXCL12 secretion

Cancer metastasis has been correlated with chemokine CXCL12 secretion and its interaction with chemokine receptor CXCR4 [18]. CXCL-12 has been identified as the substrate for MMP in cancer cells and thereby favoring the chemokine's post-translational modification

and secretion [19]. To determine the effect of violacein on chemokine expression in activated MCF-7 cells we have studied CXCL12 expression by ELISA. As shown in figure 2, there was enhanced expression of CXCL12 in activated MCF-7 cells ( $789\pm 121$  pg/mL) over basal expression ( $144\pm 97$  pg/mL,  $p<0.05$ ). Following treatment with violacein there was decreased secretion ( $293\pm 86$  pg/mL,  $p<0.05$ ) of inflammatory chemokine CXCL-12, thus strongly suggesting anti-inflammatory effect of violacein. Further, specific knock-down with MMP-2 siRNA also decreased ( $352\pm 78$  pg/mL,  $p<0.05$ ) the CXCL-12 expression which supports the contention from literature evidence that CXCL12 expression is modulated MMP-2 activity. However, vehicle (DMSO) treated activated MCF-7 cells did not demonstrate any change ( $763\pm 135$  pg/mL,  $p=0.88$ ) in the CXCL-12 expression. Cell viability was not affected upon treatment with siRNA and scramble siRNA negative control did not decrease the CXCL-12 secretion (data not shown). These data suggest that violacein directly inhibits pro-inflammatory cytokine induced MMP-2 mediated CXCL-12 secretion.

### 3.3. Violacein inhibits MMP-2 mediated MMP-9 activation

Synergistic effect of MMP-2 and MMP-9 have been implicated in the inflammatory chemokine/ligand interaction [20]. MMP-9 has been attributed in the CXCL12/CXCR4 interaction [21]. To demonstrate the effect of violacein on synergistic effect of MMP-2 and MMP-9 expression through CXCL12 action, we have performed co-culture studies. As shown in figure 3A, co-culture of activated MCF-7 cells with unstimulated MCF-7 cells induced expression of MMP-9 ( $35\pm 4$  U/ $\mu$ g) compared to non-co-culture levels ( $3\pm 1$  U/ $\mu$ g,  $p<0.05$ ). Violacein has efficiently inhibited the MMP-9 expression and activity ( $5\pm 2$  U/ $\mu$ g,  $p<0.05$ ) similar to basal level. It is important to that specific siRNA knockdown of MMP-2 and antibody neutralization of CXCL12 completely abrogated the MMP-9 expression (figure 3B and 3E). Further, directly stimulation with CXCL12 alone (without TNF- $\alpha$  and TGF- $\beta$ ) also induced MMP-9 activity (figure 3C and 3E) with minimal effect on MMP-2 activity (figure 3C and 3D). These data specifically demonstrate that cytokine inflammatory injury induced MMP-2 mediated CXCL-12 secretion is critical for the upregulation of MMP-9 activity which is efficiently inhibited violacein.

### 3.4. Violacein inhibits MMP-9 mediated CXCR4 expression

The chemokine receptor CXCR4 has been directly implicated in the cancer metastasis [21]. MMPs have been known to initiate CXCR4 expression. To demonstrate the effect of violacein on MMP-2/9 mediated CXCR4 membrane expression, we have performed flow cytometry based analyses in the co-culture studies. As shown in figure 4A, co-culture of activated MCF-7 cells with unstimulated MCF-7 cells induced expression of CXCR-4 ( $71.3\pm 9.6\%$ ) compared to non-activated levels ( $1.8\pm 0.7\%$ ,  $p<0.05$ ). Further, violacein effectively inhibited the tumor metastasis and progression marker CXCR4 ( $6.1\pm 1.4\%$ ,  $p<0.05$ ). As expected, the specific conditions, namely, MMP2 and MMP9 knockdown, and CXCL12 neutralization, have all inhibited the CXCR4 expression indicating that these molecules act upstream of CXCR4 expression. Taken together, these data precisely demonstrate that violacein can efficiently inhibit the inflammatory injury induced MMP-2/-9 mediated CXCL-12 chemokine and also inhibit its specific metastatic ligand CXCR4 expression, thus suggesting that violacein has a potential anti-inflammatory and anti-metastatic effect.

## 4. Discussion

Natural products have been regarded as attractive sources of therapeutic agents [22]. In the pharmaceutical industry, microorganisms have become an important source of natural products. Nearly 63% of commercially available drugs are directly or indirectly derived from microorganisms, plants and animals [23]. Several studies have reported the beneficial effect of violacein as an anti-parasitic, antitumor, and agent [1, 2]. Violacein is a potentially interesting therapeutic candidate as an anti-inflammatory and anti-tumor agent because of its effect towards antagonizing the pro-inflammatory cytokine effect. In our current study we specifically focused on studying the anti-inflammatory and anti-tumor metastatic properties of violacein through modulation of MMP and chemokine signaling.

Metastasis is the most critical aspect of tumorigenesis, because over 90% of cancer mortality is caused by metastasis. Recent studies unambiguously show that metastasis requires close collaboration between cancer cells and inflammatory milieu in the tumor microenvironment [24]. The process of inflammation-induced metastasis promotes tumor cell migration. Inflammation promote this tumor cell migration through production of local adhesion molecules such as CXCR4 [25]. One of these inflammatory signals involved in the activation and metastasis promotion is TNF $\alpha$  [26]. While TNF $\alpha$  plays a critical role in chemokine expression and cellular adhesion, another cytokine TGF- $\beta$  is an important regulator of the basement membrane invasion and metastasis [27]. Therefore to mimic in vivo cancer microenvironment in our current studies we have initially activated the MCF-7 breast cancer cells with the activation cock-tail consisting of TNF- $\alpha$  (50 ng/mL) and TGF- $\beta$  (50 ng/mL).

Cancer cell invasion requires extensive proteolysis of the extracellular matrix at the invasive front. Inflammatory cells are important sources of proteases that degrade the extracellular matrix. In a animal model of invasive breast cancer it has been shown that inflammatory cytokines induced cancer cells to secrete the matrix metalloproteinases MMP2 and MMP9 which promoted tumor invasion [28]. The migration of metastasis-initiating cells is not random and is directed by chemokine gradients sensed such as CXCR4 and CXCL12 interactions along with other chemokines inducing adhesion molecules [29]. CXCL12–CXCR4 interactions have been implicated in the pathogenesis and progression of breast cancer, pancreatic cancer, several types of leukemias and neuroblastomas [29]. Studies by Singh et al., in prostate cancer models have demonstrated that the CXCL12–CXCR4 interactions that also modulate MMP expression [18]. In agreement with these studies, we have shown that inflammatory cytokine activated MCF-7 cells induced upregulation of MMP2. This MMP2 has been shown to be important for CXCL12 processing and secretion. Violacein has efficiently inhibited the MMP2 upregulation (figure 1) and CXCL12 expression (figure 2). These data demonstrate a novel mechanism of violacein as an anti-inflammatory agent.

CXCR4 expression by cancer cells may also correlate with disease severity. Muller et al have showed that normal breast tissues expresses low amounts of CXCR4, whereas breast neoplastic tissues expressed high levels of CXCR4 [14]. Moreover, it has been shown that CXCL12 ligation can also mediate cancer cell adhesion to endothelial cells [30]. Studies

have demonstrated that CXCL12 induction, in prostate and breast cancer cell lines significantly express MMP-9 [18, 31]. Further, it has been shown that CXCL12 also promotes lung cancer metastasis through MMP-9 interactions [32]. In agreement with these studies, our results show that CXCL12 can increase the expression of MMP-9. Importantly, we demonstrate that violacein has inhibited this synergistic effect of MMP-2 and -9 (schematic depicted in figure 4B) and thereby potentially inhibiting the inflammation-induced cancer metastasis. Taken together, the data suggest that at early stages violacein inhibits the mild inflammation mediated MMP-2 expression induced by TNF- $\alpha$  and TGF- $\beta$ . Further the CXCL12 mediated activation of MMP-9 and eventually chemokine ligand CXCR4 interaction from different cellular source can also be inhibited by violacein. The resident MMP-2 activity is likely to be sufficient to cleave CXCL12, the extracellular secretion of which induces the chemotactic movement of the cancer cells. Similar role of MMP-2 in chemokine processing has also been reported in IL-1 $\beta$  inflammatory cytokine induced peritonitis [20]. Interesting this report by Song et al, indicates a MMP-2/-9 synergy in the CXCL5 processing resulting in neutrophil infiltration [20]. Our studies further extend this study to reveal MMP-2/-9 synergistic action to promote the interaction of CXCL12-CXCR4 which is efficiently abrogated by violacein.

Based on our studies, we conclude the violacein exerts potent anti-inflammatory and anti-tumor activity through inhibition of MMPs mediated CXCL12-CXCR4 interaction. The development of violacein to treat cancerous disease is limited, however, by a lack of rational insight into the molecular mechanisms by which violacein affects MCF-7 cells could be extended in future to study its effect in other solid tumors and animal models. Our current studies provide basic molecular basis of violacein mechanism of action can be studied further as a potentially novel anti-cancer agent.

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## Abbreviations

<b>TNF<math>\alpha</math></b>	Tumor Necrosis Factor alpha
<b>TGF<math>\beta</math></b>	Tumor Growth Factor beta
<b>MMP</b>	matrix metalloproteinase
<b>MCF-7, human breast adenocarcinoma cancer cell line</b>	Michigan Cancer Foundation
<b>CXCL12</b>	chemokine C-X-C motif ligand 12
<b>CXCR4</b>	chemokine C-X-C motif ligand receptor 4

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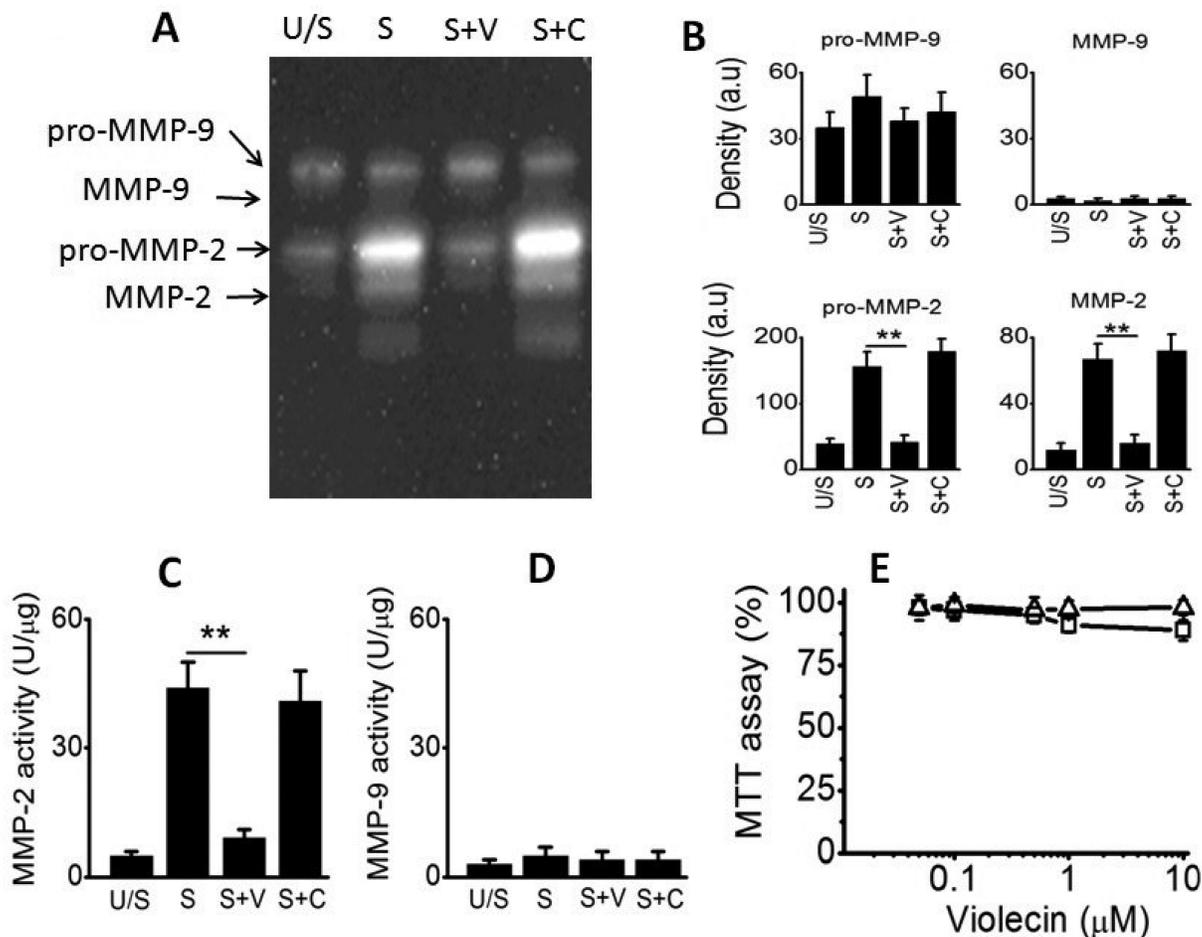
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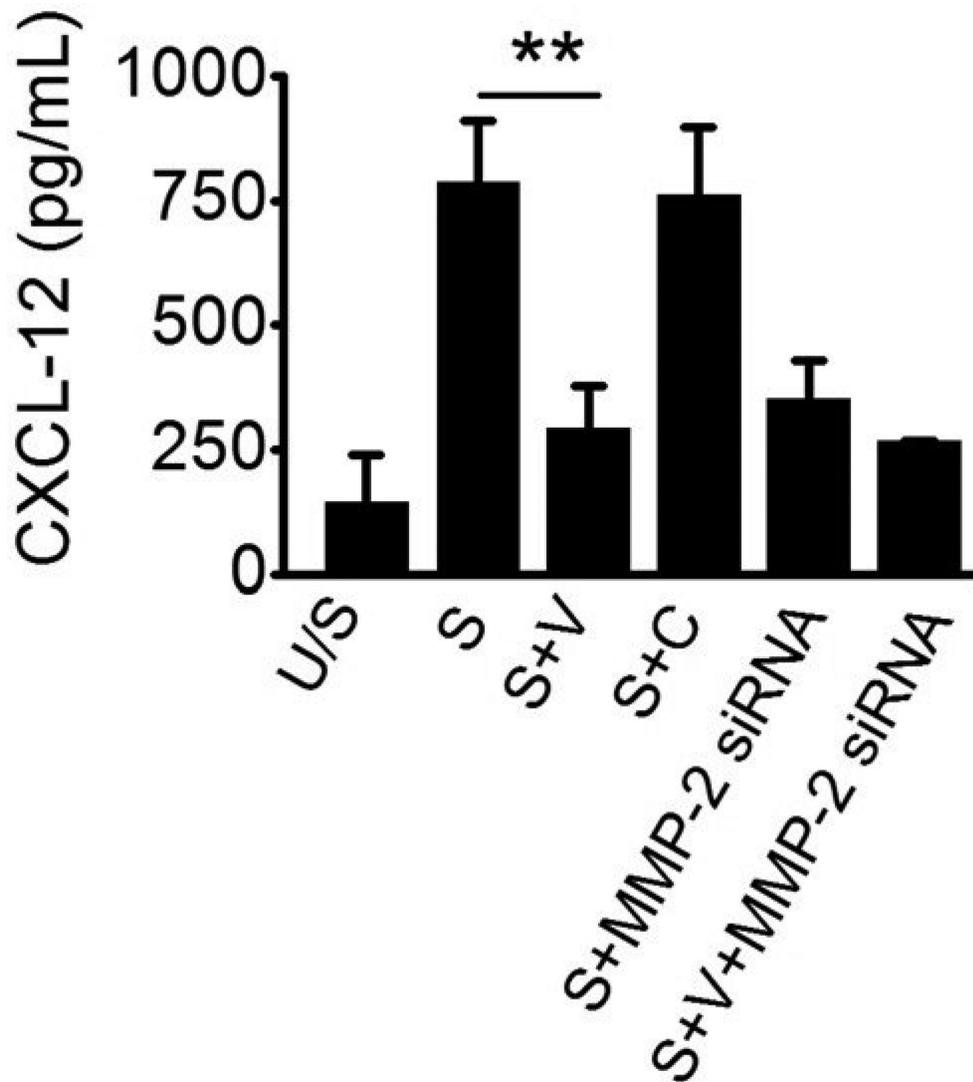
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### Research Highlights

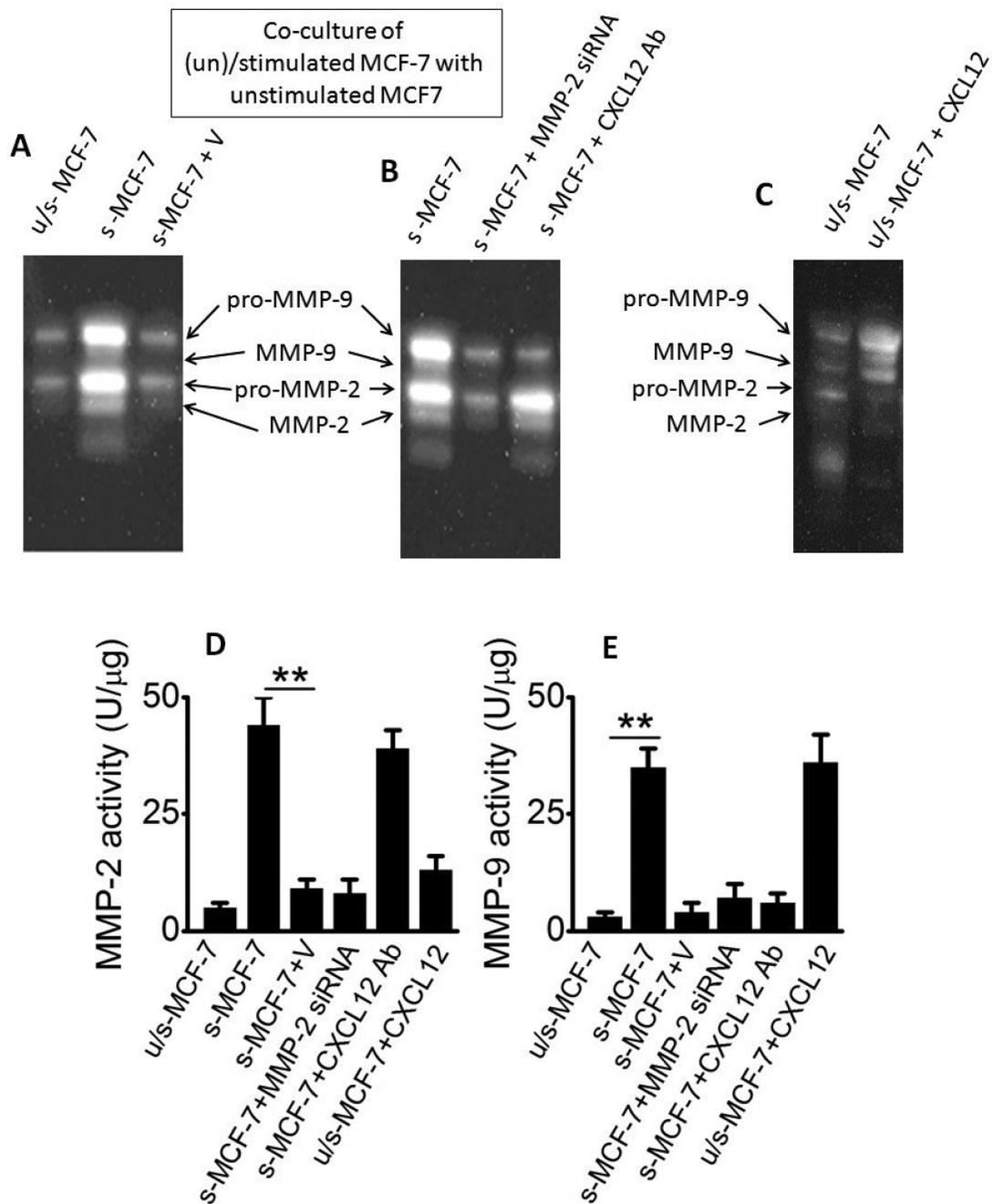
- CXCL12/CXCR4, chemokine-ligand interaction plays a critical role in tumor metastasis.
- MMP-2 and -9 act synergistically in the upregulation of CXCL12/CXCR4 expression.
- Violacein decreased CXCL12 secretion through MMP-2 inhibition.
- Violacein diminished CXCR4 membrane expression through MMP-9 inhibition.
- Therefore, violacein could represent a novel anti-metastasis drug in cancer therapy.

**Figure 1.**

Inhibition of MMP-2 activity by violacein. (A) Gelatin zymography assay to determine the activity of (pro-)/MMP2 (66 kDa) and (pro-)/MMP9 (97 kDa) under following conditions: (U/S) basal unstimulated, (S) stimulated with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TGF- $\beta$ , (S+V) treatment with 1  $\mu$ M violacein in DMSO along with stimulation with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TGF- $\beta$ , (S+C) treatment with vehicle control DMSO along with stimulation with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TGF- $\beta$ ; (B) Densitometric analysis of the zymograms in A for the pro-MMP-2/9 and activated MMP-2/9; (C and D) Gelatinase substrate activity assay of MMP2 (C), and MMP9 (D) were performed in the above mentioned groups; (E) MTT assay to determine the cell viability assay of MCF-7 cells upon treatment with violacein (0-10  $\mu$ M), (○) treatment with vehicle control DMSO, (□) treatment with violacein in DMSO; (\*\*\*)  $p < 0.05$ .



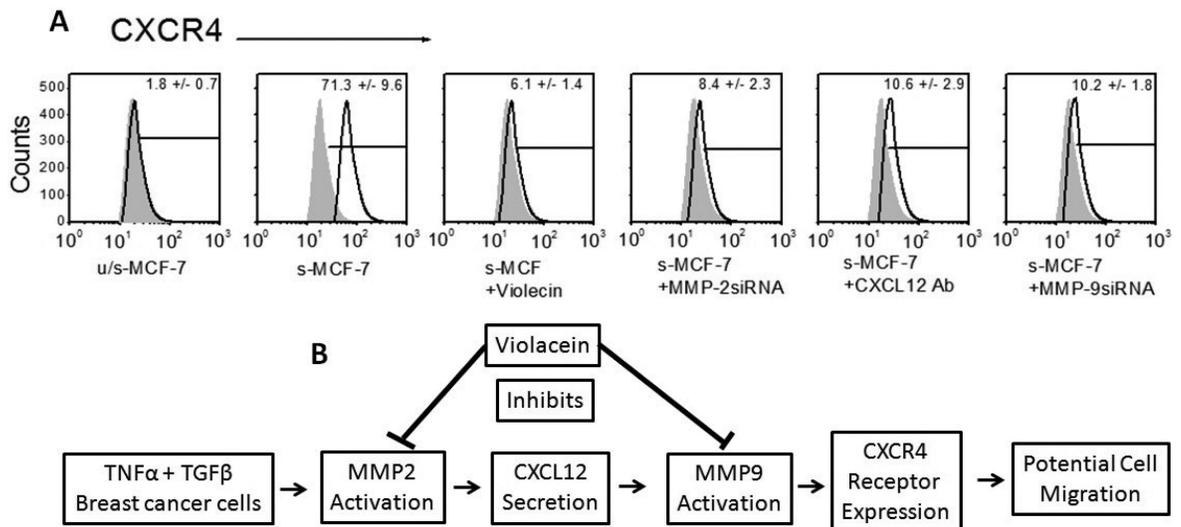
**Figure 2.** Inhibition of MMP-2 mediated CXCL-12 secretion by violacein. ELISA analysis of CXCL-12 secretion from MCF-7 cells under following conditions: (U/S) basal unstimulated, (S) stimulated with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ , (S+V) treatment with 1  $\mu$ M violacein in DMSO along with stimulation with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ , (S+C) treatment with vehicle control DMSO along with stimulation with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ , (S+MMP-2 siRNA) treatment with MMP-2 siRNA in DMSO along with stimulation with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ , (S+MMP-2 siRNA) treatment with 1  $\mu$ M and MMP-2 siRNA along with stimulation with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ , (\*\*) $p < 0.05$ .



**Figure 3.**

Inhibition of MMP-2 mediated MMP-9 activation by violacein. (A) Co-culture of unstimulated MCF-7 cells - with (u/s-MCF-7) unstimulated MCF-7; (s-MCF-7) with MCF-7 cells stimulated with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ; (s-MCF-7 + V) 1  $\mu$ M violacein treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ . Co-culture of unstimulated cells with stimulated cells induced MMP-9 activity along with previously activated MMP-2 in stimulated cells. (B) Co-culture of unstimulated MCF-7 cells - with (s-MCF-7) with MCF-7 cells stimulated with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ;

(s-MCF-7 + MMP-2 siRNA) MMP-2 siRNA treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ; (s-MCF-7 + CXCL-12) CXCL-12 blocking antibody (1:100) treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ . Co-culture of unstimulated cells with stimulated cells under MMP-2 knock-down and CXCL-12 blocking did not result in MMP-9 activation. (C) Co-culture of unstimulated MCF-7 cells with (u/s-MCF-7) unstimulated MCF-7; (u/s-MCF-7 +CXCL12) CXCL-12 (3 ng/mL) treated to unstimulated MCF-7. Co-culture of unstimulated cells with CXCL-12 cytokine resulted in MMP-9 activation with no effect on MMP-2 activity. (D and E) Gelatinase substrate activity assay of MMP2 (D), and MMP9 (E) were performed in the above mentioned groups, (\*\*)  
p<0.05.



**Figure 4.**

(A) Inhibition of CXCR4 receptor expression by violacein. Flow cytometry analysis of CXCR4 expression under the following co-culture conditions: co-culture of unstimulated MCF-7 cells - with (u/s-MCF-7) unstimulated MCF-7; (s-MCF-7) with MCF-7 cells stimulated with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ; (s-MCF-7 + V) 1  $\mu$ M violacein treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ; (s-MCF-7 + MMP-2 siRNA) MMP-2 siRNA treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ; (s-MCF-7 + CXCL-12) CXCL-12 blocking antibody (1:100) treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ ; (s-MCF-7 + MMP-9 siRNA) MMP-9 siRNA treated stimulated MCF-7 cells with 50 ng/mL TNF- $\alpha$  and 50 ng/mL TNF- $\beta$ . As shown CXCR4 expression was inhibited by violacein and also, by MMP-2 siRNA, CXCL-12 blocking, and MMP-9 siRNA, individually, indicating this are all upstream to CXCR4 expression pathway. (B) Schematic for the proposed mechanism of action of violacein.