

**Breast Cancer, Chemokines, And Metastasis: A Search for Decoy Ligands of the CXCR4 Receptor**

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**Abstract:**

Breast cancer (BC) is the leading cause of cancer-related deaths in young to middle-aged women worldwide. Moreover, the survival rate in BC-patients is only 20% when associated with metastatic disease. The high mortality rate observed in BC women with metastatic disease has precipitated a major challenge revealing an unmet need to develop new therapeutic strategies in treating metastatic cancer. One such approach has involved utilization of chemokines and their receptors as therapeutic targets for cancer metastasis. It has been established that a definitive correlation exists between overexpressed CXCR4 malignant cell receptors and cancer cell growth, invasion, and migration. It is also widely accepted that the CXCR4 receptor, complexed to its CXCL12 ligand, plays a major role in establishing migratory pathway gradients for cancer cells migrating to distant tissues/organ sites. It would follow that chemokine decoy ligands, such as peptide antagonists and inhibitors, could serve to induce receptor blockade and impede subsequent intracellular signaling. Such ligands, synthetic and natural, reportedly contribute to reducing cancer cell growth, invasion, adherence, and migration. The present commentary describes several existing synthetic CXCR4 receptor-ligand peptide antagonists and presents a strategy to develop naturally-occurring human protein-derived peptide candidates.

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## Introduction

Breast cancer (BC) poses the highest incidence among cancer types in women and accounts for 30% of all new cancers in females worldwide [1]. BC is also the leading cause of cancer-related deaths in women especially between ages of 20 to 50 years [2]. When associated with extensive metastasis, BC-associated mortality rises to 80 - 90%. Hence, there exists major unmet needs in treating metastatic disease in BC patients, even though new chemo-therapeutic drugs are constantly being developed and assayed for efficacy. The survival rate in patients with BC metastatic disease approximates 20% of patients after 5 years [3]. Thus, most cancer deaths can be attributed to metastasis rather than the primary tumor mass itself. The present commentary addresses this unmet need by discussing chemokine receptors and ligands as therapeutic targets for cancer growth and metastasis.

Tumor cell metastasis appears to comprise five sequential steps as follows [2, 4]. First, tumor cells shed from the primary tumor mass infiltrate and invade into local surrounding stromal cell extracellular matrix spaces and penetrate the basement membranes of nearby vasculature. Second, the migrant cells intravasate into the microvasculature lumen of nearby blood vessels and lymphatic ducts. Third, the now circulating tumor cells (CTCs) manage to aggregate into micro-cell clusters and hide among platelets aided by a process termed tumor cell-induced platelet aggregation (TCIPA). The TCIPA step, first described by Gasic et al, is a common occurrence but often overlooked component of the cancer cell metastatic process [5]. Tumor cells in the vasculature are commonly observed in complexes with platelets which serve to avoid immune surveillance, a cloaking trait widespread among circulating tumor cancer cells (CTCs) including BC. Fourth, the RNA transcriptome of CTCs has recently been reported revealing that such cells exhibit a semi-dormancy state bent on intravascular survival and maintenance of sufficient cell signaling pathways to maintain basic cell functioning [6, 7]. The fifth step involves extravasation of CTCs from the blood and lymphatic vessels into distal target tissues/organs, nesting into the host connective tissue stroma and adapting to local microenvironments. Certain tissue/

organ-derived metastatic cells appear to show destination preferences depending on tumor type, with BC cells favoring liver, lungs, bone marrow, and brain. Therefore, tumor metastatic cells scattered among multiple distal organs present an enormous challenge to clinical therapists attempting to treat metastatic disease in cancer patients.

## Chemokines, Receptors, and Breast Cancer

Chemokines are a subgroup of peptides termed cytokines (5-20 kD) which serve as immunomodulating agents for autocrine, paracrine, and endocrine signaling functions [8]. Chemokines are chemo-attractive cytokines that regulate many cellular functions including cellular homing, migration, differentiation, homeostasis, survival, and trafficking [9]. Chemokines especially mediate the migration of cells into and out of normal tissues; unfortunately this includes metastatic cancer cells as well. This present commentary will focus on a subfamily of chemo-attractant cytokines termed the CXC chemokines, namely, the chemokine ligand CXCL12 originally known as stromal-derived factor-1 (SDF-1) [10]. CXCL12 ligand is normally expressed on stromal cells, fibroblasts, thymic cells, and endothelial cells of blood vessels and lymphatics. It is further expressed on multiple cancer cells derived from breast, ovary, prostate, colon, liver, and others [11, 12]. Interestingly, many non-malignant (normal) cells from tissue/organs such as breast do not express CXCL12 chemokine ligands and their receptors; in contrast, breast cancer cells overexpress both [13]. The CXCL12 serves as the ligand for the chemokine receptors CXCR4 and CXCR7 which are both G-coupled cell membrane receptors. The G-coupled receptors display the canonical seven-transmembrane spanning domains and are linked to the Gai and Gq associated GTPases [14]. The CXCR4 receptor is a 352 amino acid rhodopsin-like G-coupled protein that selectively binds the CXCL12 chemokine as its cognate ligand. The physiological role of CXCR4 in embryonic and adult tissues is to promote cell proliferation, homing, migration, homeostasis, and trafficking in many cells including T-cells in lymphoid organs and hematopoietic stem cells in bone marrow [15]. The binding and interaction of CXCL12 to its CXCR4 receptor leads to activation of multiple intracellular signal transduction pathways and

downstream effector cascades. Such signaling pathways include not only PI3K/AKT, Src/ERK1-2, NF- $\kappa$ B, and STATE-3, but also cross-talk between CXCR4 and NOTCH, Wnt, and SHH networks [10]. These signal cascade networks can promote cell growth, proliferation, migration, adhesion, and chemotaxis in both normal and cancer cells including metastatic cells [16]. The latter activities are especially crucial regarding initial cell detachment from primary tumor masses and subsequent migration via CXCL12/ CXCR4 (ligand/receptor) induced trans-endothelial passage into the bloodstream [17].

The primary objective in the present commentary is to search and identify short peptides that could pose as chemokine mimics and serve as decoy ligands to bind and deactivate signal transduction of the CXCR4 receptor. Decoy ligands are defined as ligands that bind to receptors, but do not activate the receptor signaling cascades resulting in receptor blockade and functional neutralization. Such a blockade, if sustained and long-term, eventually leads to receptor de-sensitization via the Beta-Arrestin pathway resulting in down-regulation (expression) of the receptor [18] (see below). In contrast, the over-expression of CXCR4 receptor and its CXCL12 ligand greatly enhances both cancer cell growth and migration leading to metastasis [13, 19, 20].

### **Requirements for a Decoy Ligand of the CXCR4 Receptor**

To date, it is well-established that a positive correlation exists between CXCR4 receptor up-regulation and malignant tumor growth, angiogenesis, invasion, and migration [2, 10]. Thus, the CXCR4/CXCL12 axis is widely accepted as a critical therapeutic target for breast and other cancer cell metastasis. Indeed, several synthesized and natural receptor antagonist ligands have already been developed and some are FDA-approved to block CXCR4 receptor activation and metastasis of multiple solid tumors including breast cancer [21]. At present, few if any, CXCR4 peptide antagonist/inhibitors have survived clinical trials and achieved clinical status for treating human tumor metastasis. Several reasons exist for this shortcoming, namely, a) the peptide inhibition of over-expressed CXCR4 has been produced, but without providing a means to prevent abnormal

regulation of CXCR4 signal transduction cascades in non-malignant cells; and b) CXCR4 peptide antagonists can cause inhibition of host immune cell function (i.e. preventing T-cell cytotoxicity of tumor cells); and c) blocking the migration of normal hematopoietic stem cells to distant organs/tissues. As discussed above, chemokines and their receptors mediate normal cell homing and migration of cells into and out of tissues. However, recently developed peptide antagonists/inhibitors can often block normal (global) functioning and induce aberrant signaling of CXCR4 receptors in the host body.

Although there already exists several peptide antagonist (inhibitors) of the CXCR4 receptor, the blocking of normal receptor functioning poses an unacceptable tradeoff. It can be posited that the ideal decoy ligand for CXCR4 should display and/or possess certain characteristics in addition to the basic ones described above. First, the decoy ligand should not block the global functioning of the chemokine receptor throughout the host body. Second, the peptide decoy should not suppress the immune system functioning of the host, especially T-cell cytotoxicity against tumor cells. Third, the antagonist ligand should be capable of inhibiting tumor cell growth, proliferation, and migration. Fourth, the decoy ligand need not suppress cellular expression of the CXCR4 receptor because continuous long term de-sensitization of the receptor will accomplish this. Fifth, the peptide decoy should be capable of suppressing tumor cell invasion, mobility, and migration while not interfering with normal cell contact, spreading, and adherence. Sixth, the receptor antagonist should not be toxic nor produce ill side-effects or off-target bystander cell damage. Seventh, the decoy ligand should be anti-angiogenic in order to inhibit formation of new blood vessels within and about the primary tumor. Eighth, it would be advantageous if the peptide antagonist were able to disrupt the cell membrane lipid bi-layer to increase membrane fluidity (thinning) thus interfering with cell surface chemokine receptor clustering/aggregation and subsequent intracellular signal transduction [22].

### **Synthetic Peptide Decoy Ligands for CXCR4**

Several synthetic peptide antagonist/inhibitor

decoy ligands for CXCR4 receptor have previously been developed as HIV-entry inhibitors (see below) and their activities reported in the literature. One such synthetic peptide ligand inhibitor is termed "E5" which is a 22-amino acid chemo-sensitizing and anti-angiogenic peptide able to reduce tumor cell migration and adhesion [23]. A second peptide antagonist termed Plerixafor (AMD3100) was reported to decrease the metastatic potential in animal cancer models [24]. Peptide T140 is a decoy receptor ligand which was reported to suppress tumor cell invasion into surrounding cells [25]; while a TN14003 peptide was shown to inhibit both tumor cell invasion and migration [26]. A further synthetic peptide antagonist termed BKT140 reportedly reduced extra- and intra-tumor vascularization. Finally, the peptide decoy ligand LY2510924 was found to suppress the growth of several different cancer types [27]. However, few if any of the peptides were capable of reaching clinical therapeutic status, even though some achieved two or more of the metastatic suppressive characteristics listed above. None of the developed peptide decoy ligands provided evidence to prove that normal (global) whole body CXCR4 function was not impaired, even those employing animal models. Most of the peptides assayed were cytotoxic in mechanism of action, some displayed weak agonist activity, while others required high peptide concentrations. A few demonstrated unwanted side effects, very short half-lives, but most lacked oral availability. In general, some peptide antagonists (i.e. T140) were quite effective in reducing metastases by inhibiting migration and tumor cell growth but lacked action against tumor angiogenesis. Finally, human clinical trials using BL-8040 and POL 6323 as antagonist of the CXCR4 receptor are currently in progress and can be pursued by interested readers. Thus, the ideal peptide antagonist/inhibitor should at the very least: a) avoid blocking normal CXCR4 function such as the mobilization and movement of normal cells from tissue origins and b) not suppress tumor-associated T-cell cytotoxicity function.

### **Naturally-occurring Decoy Ligands for CXCR4 Receptors**

Although naturally occurring antagonists/inhibitors for the CXCR4 receptor have been reported

(see above), all have been root, stem, and bark extracts from plants such as ginseng, cashews, horny goat weed, and terpine resin derivatives such as boswellic acid [10]. To date, with the exception of the viral macrophage inflammatory protein-II, no peptide decoy ligands from naturally-occurring mammalian (including human) proteins have been developed, reported, or described. Nevertheless, short human peptide fragments are known to manifest a variety of biological activities. Some peptide segments are often buried in hydrophobic clefts of folded proteins while others are exposed on linear stretches or near bends of the tertiary fold. Thus, human protein-derived short peptide fragments could represent a little-known untapped reservoir of molecules encrypted within proteins representative of growth factors, extra-cellular matrix and blood proteins, clotting factors, and adhesion and angiogenic proteins. These protein-encrypted peptide segments can affect and/or modulate activities such as growth regulation and proliferation extra- and intracellular signaling, angiogenesis, cell migration, and adhesion. The short peptides can be chemically-synthesized as single fragments of 8-35 amino acids, or be found exposed on a protein exhibiting a conformational change or a slightly denatured phase transition. Naturally-occurring human protein-derived peptides are now potentially undescribed decoy ligands for various receptors including chemokines. However, pharmaceutical companies have been reticent in developing short peptides as medicines due to: a) cost of synthesis b) lack of mass production and inexpensive delivery systems c) and an industry-wide lag in acceptance of peptides as cost-effective drugs.

Alpha-fetoprotein (AFP) is tumor-associated fetal protein present during pregnancy and in certain adult cancer types [28, 29]. Short peptides derived from the full-length AFP polypeptide have been reported to display diverse biological activities related to suppression of tumor growth, adherence, migration, and metastasis [29, 30]. Reports of the binding and interaction of AFP itself with chemokine receptors have been known since 2002 when AFP was demonstrated to bind both CCR5 and CXCR4 as co-receptors that promote HIV transmission at the cell surface of

monocytes, CD4 T-cells, and macrophages [31, 32]. A subsequent study using computer modeling software followed by cell-based assay confirmation demonstrated that certain AFP-derived peptides were capable of binding to several chemokine receptors (especially the CXCR4 receptor) at short AFP amino acid segments [33]. One such AFP peptide sequence on the AFP third domain fragment has been isolated, purified, characterized, and assayed for biological activity [34, 35]. This 34-amino acid peptide and its subfragments have been termed the Growth Inhibitory Peptides (GIP) and were found to interact with the CXCR4 receptor [34]. In the present commentary, AFP-derived-peptide fragments are presented as example peptide decoy ligands for the CXCR4 receptor for use on both primary tumor cells and their metastatic counterparts. In addition to the 34-mer GIP, the author (GJM) has previously developed a 9-mer subfragment of GIP termed P149c, an effective inhibitor of estrogen (E2)-induced cancer growth [35, 36]. Later, subsequent investigators modified P149c into a 9-mer cyclic peptide named AFPep, which also suppressed E2-induced cancer growth [37]. However, unlike GIP, both P149c and AFPep lack significant anti-metastatic capabilities [38-40].

### **Biological Activities of AFP-derived Peptide Fragments**

Numerous publications over the last two decades have demonstrated that AFP-derived peptides (AFP-DPs), such as GIP have demonstrated many of the biological effects which could blunt, obstruct, and possibly impede the spread of CTCs in BC patients with metastatic disease [38-44]. The biological activities of AFP-DPs could potentially disable CXCR4 receptor intracellular signaling pathways and downstream cascading possibly without disrupting the non-malignant cell functioning of CXCR4 (see below). First and foremost, GIP has been demonstrated to inhibit the growth of primary BC cells in both in vitro and in vivo models while displaying a cytostatic mechanism of action utilizing cell cycle arrest and inhibitor protein (i.e., p27) preservation [45, 46]. The cytostatic activity of GIP stands in dire contrast to of the peptide antagonist/inhibitors of CXCR4 which result in cytotoxic destruction of the cancer cells and deleterious side effects.

Furthermore, AFP-DPs show no bystander and off-target cell demise. AFP-DP reports have not demonstrated any upper toxic dose levels and no side effects as described in mouse studies [36-38]. Second, AFP-DPs (i.e., GIP) do not suppress immune function, but rather enhance the immune response as shown in Concanavallin-A lectin-induced blast transformation in vitro assays; in addition, AFP-DPs can provide several antigenic sites for initiating cell-mediated immunity against tumors [38, 39]. Third, AFP-DPs were found to be effective anti-angiogenic factors as reported using the chick embryo chorio-allantoic membrane (CAM) assay and in studies employing tumors implanted onto chick egg shell membrane blood vessel sites [38-40]. Fourth, the AFP-derived peptides were demonstrated to display molecular mimicry of several chemokine ligands (including CXCL12), implying a potential role of AFP-DP as antagonist/inhibitor ligands [33]. Regarding cell migration, AFP-DPs can inhibit cell spreading, extra-cellular matrix adherence, and affect BC cell-to-cell contact inhibition [33, 34, 39]. Furthermore, some GIP and subfragment peptides perturb the lipid cell membrane bi-layer and are known as cell membrane disruptive agents. This activity results in a ruffling of the plasma membrane causing a) increased membrane fluidity, b) thinning of the cell membrane, and c) a reduction of receptor aggregation and oligomerization at the cell surface [46, 47].

AFP-DPs, specifically GIP, also differ from other peptide CXCR4 antagonists being reported to be both radio-sensitizing and chemo-sensitizing agents to enhance apoptosis in targeted cells following exposure to chemo-drugs and gamma radiation in the presence of  $10^{-8}\text{M}$  to  $10^{-10}\text{M}$  GIP [38-40]. A further advantage of GIP as a CXCR4 blocking agent is that it can inhibit platelet aggregation by 95% in assays employing adenosine diphosphate, arachidonic acid, or collagen-III as stimulators [38, 39]. In effect, this activity could serve to reduce or hinder tumor cell-induced platelet aggregation, thus preventing intravascular tumor cell clustering. In contrast, neither P149c or AFPep had any effect on platelet aggregation [38]. GIP might also promote immune surveillance (T-cells) in the bloodstream to uncloak and expose tumor cells to lymphocytic attack, thus impeding metastatic spread. In related studies, the estrogen (E2) receptor has been

reported to play a major role in promoting the expression of CXCR4 by inducing post-translational modification of the chemokine receptor, which is apart from directly inducing CXCR4 transcription in BC cells [41, 48]. Conjointly, it was reported that GIP, not only suppressed E2-induced growth in BC cells, but inhibited estradiol (E2) from binding to the E2 receptor itself [49]. A further study reported that GIP fragments block BC cell adherence to multiple extra-cellular matrix (ECM) proteins during tumor cell migration [39-40]. Tumor cells are known to invade and travel through the ECM spaces using tank-like traction movements to enhance mobility. In this regard, it was found that AFP-DP fragments were capable of inhibiting 40 to 50% of tumor cell adhesion to multiple ECM cell proteins via binding to collagen IV, fibrinogen, fibronectin, thrombospondin, laminin, and vitronectin [38, 39, 46]. In that same study, GIP was reported to inhibit tumor cell migration and cell spreading by 60% in BC cell cultures [38]. Thus, AFP-DPs have been previously implicated in multiple cell surface membrane activities and in tumor cell-to-ECM adhesion enhanced by means of integrin-protein interactions with basement membranes, interstitial cell surfaces, and connective tissues [38, 39, 50].

Repeated decoy ligand binding to a cell membrane receptor and subsequent lack of activation eventually results in receptor de-sensitization via arrest of the receptor-mediated endocytotic pathway; this results in a down-regulated expression of the CXCR4 receptor on tumor-bearing cells [51]. Finally, certain AFP-DPs (specifically GIP) are amphipathic peptides capable of binding to lipid-inverted apoptotic-destined tumor cells (not normal cells) flagged for destruction by cytotoxic lymphocytes [50, 51]. Lipid inversion of polar head groups (phosphatidylserine, phosphoglycerol) in the bi-lipid cell membrane layer results in a net negative charge at the tumor cell membrane surface in contrast to the positively charged membranes of normal cells not flagged for apoptosis [52-54]. To date, AFP-DPs have not been reported to block the normal functions of CXCR4 receptors on non-malignant normal cells.

#### **Concluding Remarks:**

There exists a critical need for therapeutic strategies capable of disrupting and dispersing CTCs

intended for metastatic spread to targeted host organs. The chemokine receptor CXCR4 has been highlighted as a key player in the gradient channeling of disseminated tumor cells onto their metastatic pathway toward distant organs. Furthermore, the CXCL12/CXCR4 axis has been identified as a major factor in the promotion of BC cell growth, progression, angiogenesis, invasion, adherence and migration. The CXCL12/CXCR4 complex is now widely accepted as a prime therapeutic target to impede BC metastasis to distal organs. Attempts to design and develop peptide decoy ligands as antagonist (inhibitors) of CXCR4 function have met with a host of emerging synthetic peptide candidates representative of a "promise unfulfilled" group that failed to achieve clinical therapeutic usage. This is partially due to their propensity to interfere with CXCR4 intracellular global signaling and blockade of non-malignant cell functions. Although the synthetic "designer peptides", and natural plant extracts are capable of inhibiting some functions of CXCR4 receptors, few if any are able to block global receptor expression/signaling without interfering with normal cell homing and migration activities. Naturally-occurring protein derived peptides [i.e. GIP] have been forwarded as potential candidate decoy ligands for CXCR4 that might fulfill some functions not always addressed by synthetic peptide antagonist ligands. AFP-DPs might be capable of serving as decoy ligands for use in receptor blockade and neutralization by providing additional antagonist/inhibitor activities to the existing armamentarium of synthetic peptides designed to impede metastasis.

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