CXCR4 Inhibitors: Tumor Vasculature and Therapeutic Challenges

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Abstract: CXCL12, also known as SDF-1, is the single natural ligand for chemokine receptors CXCR4 and CXCR7. CXCL12 has angiogenic properties in normal endothelial tissue and is involved in the outgrowth and metastasis of CXCR4 expressing tumors. Recent investigations have indicated that CXCL12 levels increase after chemo- and anti-VEGF therapy, favouring recurrences. The blockade of CXCL12/CXCR4 axis has emerged as a potential additional or alternative target for neo-adjuvant treatments. We have reviewed recent patent applications between 2008 and 2011 in tumor angiogenesis and the most clinical data supporting the potential use of anti-CXCR4 agents in this field. Among these, AMD3100, also known as Plerixaform (Mozobil® by Genzyme), is approved for stem cell mobilisation in patients with leukaemia, while BKT140 (Emory University), POL6326 (Polyphor Ag) and TG-0054 (ChemoCentryx) are currently in clinical trials in combination with chemotherapy for multiple myeloma and leukaemia. The aptamer Nox-A12 (Noxxon) is in trials for chronic lymphatic leukaemia treatment. MSX-122 (Metastatix) is in Phase I trials for solid tumor treatment, while CXCR7-specific inhibitor CCX2066 (ChemoCentryx) is still in preclinical studies. We have also considered other strategies, such RNA interference and miRNA, which could be tested for solid tumor adjuvant therapy.

Keywords: Angiogenesis, cancer, CXCL12, CXCR4, metastasis, vasculogenesis.

INTRODUCTION

Tumor growth requires an extensive capillary network to provide the necessary nutrients and oxygen [1, 2]. However, intra-tumoral blood vessels offer a way for cancer cells to enter into circulatory blood system, metastasizing to distant organs [1]. Neo-vascularisation consists of two distinct processes: angiogenesis and vasculogenesis. In the angiogenesis mechanism, endothelial cells (ECs), also known as "tip cells", sprout and grow from pre-existing microvasculature Fig. (**1**). Instead, vasculogenesis is a *de novo* blood vessel development, formed by recruitment and differentiation of endothelial progenitor cells (EPCs) and various bone marrow-derived endothelial progenitor cells (BMDEPCs) through pro-angiogenic factor influence Fig. (**2**). EPCs, a subpopulation of the mononuclear cell fraction in peripheral blood, can derive from heamatopoietic stem cells (HSCs) or alternatively from the endothelium itself. HSCs can differentiate into hematopoietic progenitor cells (HPCs), which in turn give rise to lymphoid progenitor cell, the myeloid progenitor cells, and likely EPCs [3, 4]. EPCs are strongly mobilised from bone marrow (BM) in response to several pro-angiogenic factors such as chemokine ligand 12 (CXCL12) and vascular endothelial growth factor (VEGF), secreted by tumor microenvironment [5]. Results from anti-angiogenic therapy targeting VEGF pathways showed a modest overall survival in patients, indicating that other signals overcome VEGF blockade, supporting vessel sprouting [6, 7]. In this scenario, CXCL12 and its receptors, chemo-kine receptor 4 (CXCR4) and chemokine receptor 7 (CXCR7), have become an important investigation area to target blood supply in cancer therapy.

Chemokines are small secreted proteins (8-14kDa) classified according to the position of their highly conserved aminoterminal cysteine residues [8]. By binding CXCR4, a G protein-coupled receptor, CXCL12 or SDF-1 activates several mechanisms such as transcription, migration and cellular survival via different signal transduction pathways [9]. Less is known about CXCL12/CXCR7 pathway [10].

CXCL12-CXCR4 AXIS AND ANGIOGENIC PATHWAY

Role in Normal Endothelium

The complexity of CXCR4/CXCL12 signalling is still not completely understood, but many *in vitro* and *in vivo* systems indicate a key role in angiogenic pathway. *In vitro* model for early human vasculogenesis showed that both CXCL12 and CXCR4 are simultaneously expressed during EC differentiation. In addition, CXCR4 activation stimulates both EC migration and organisation into vascular network [11]. This pathway is inhibited by blocking CXCR4 signal-

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ling, while exogenous CXCL12 administration stimulates angiogenesis *in vitro* and *in vivo* systems through the expression of VEGF mRNA [12]. In the adult, CXCR4 is widely expressed on EPCs, T-lymphocytes, B-lymphocytes, monocytes and macrophages, neutrophils and eosinophils as well as in brain, lung, colon, heart, kidney, and liver. In general, CXCR4 is also expressed on embryonic pluripotent and tissue-committed stem cells [13]. In contrast, CXCL12 is constitutively secreted by BM stromal cells, mainly osteoblasts, where it creates a microenvironment in which HPCs and EPCs are retained [14-16]. In this "niche" the physical interaction between stem cells and BM stromal cells allows survival and differentiation during haematopoiesis [16]. However, all these cells may be mobilised from BM by locally increased concentrations of CXCL12 [17]. Hypoxic conditions such as tissue damage, heart infarct, limb ischaemia, atherosclerotic plaques, toxic liver damage and excessive bleeding, increase CXCL12 secretion from injured site via hypoxia transcription factor 1 (HIF-1) activation. CXCL12 binding CXCR4, supports physiological events such as inflammation, tissue repair and ischemic tissue revascularisation via EPC recruitment [18] Fig. (**1**).

Tumor Vascularisation: Preclinical Studies

Several extensive studies investigated the role of CXCR4 in haematopoiesis, especially in stem cell mobilisation for treatment of leukaemia, in which CXCR4 blockade represents a valid adjuvant therapeutic approach [19]. In solid tumors, several stromal cells secrete high CXCL12 levels and both CXCR4 and CXCR7 are markedly overexpressed [20-22]. Furthermore, some studies have shown a direct correlation between CXCR4 upregulation and tumor growth/progression, neovascularisation, invasion and metastatisation [23-25]. On binding CXCR4, CXCL12 may, in fact promote cell proliferation and survival via AKT pathway [10]. Moreover, CXCL12 may support tumor growth stimulating vessel formation by recruiting BMDEPCs, such as $CD11b^{+}$ myelomonocytes (with pro-angiogenic activity) [26], and EPCs [18, 27] Fig. (**2**). This finding was validated by the observation that coinjection of breast cancer cells and carcinoma-associated fibroblasts (CAFs) in mice led to highly vascularised tumors. In contrast, poorly vascularised tumors were generated by coinjection of tumor cells with normal stromal fibroblasts [28].

Furthermore, stress conditions in the tumor microenvironment such as low glucose levels, radical oxygen species, hypoxia and tissue damage after irradiation or chemotherapy, may upregulate CXCR4 and CXCL12 via activation of transcription factors. In particular HIF-1 increases CXCR4 transcription by binding its promoter [29]. HIF-1 can induce a local expression of several molecules such as VEGF its receptor (VEGFR1), placenta growth factor (PlGF) and CXCL12, may enhance BMDC recruitment or stimulate EC sprouting [26, 30] Figs. (**1** & **2**). In breast cancer cells, CXCL12 may induce an "angiogenic switch" trans-activating HER2/neu by a pathway involving Src kinase activation. In prostate cancer angiogenesis is promoted by upregulation of VEGF, IL-6, IL-8 and tissue inhibitor of metalloproteinases

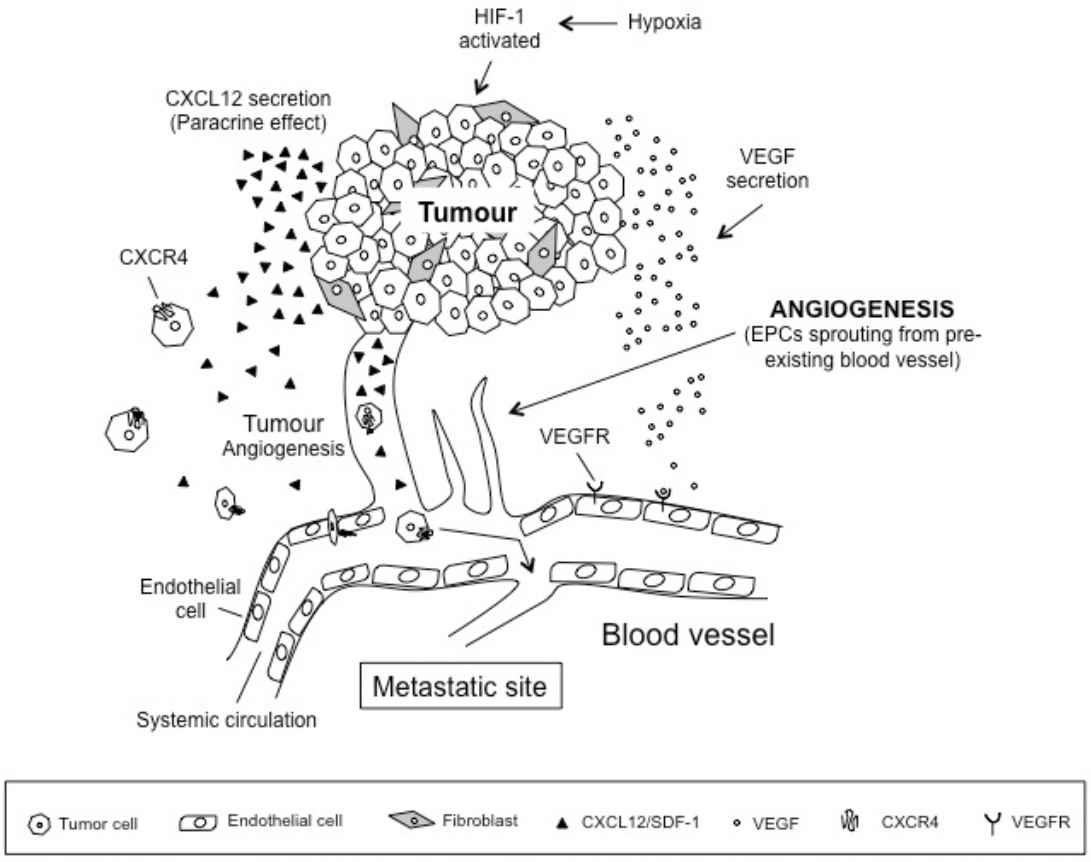

Fig. (1). Tumor angiogenesis. Schematic representation of CXCL12/CXCR4 axis and sprouting mechanism.

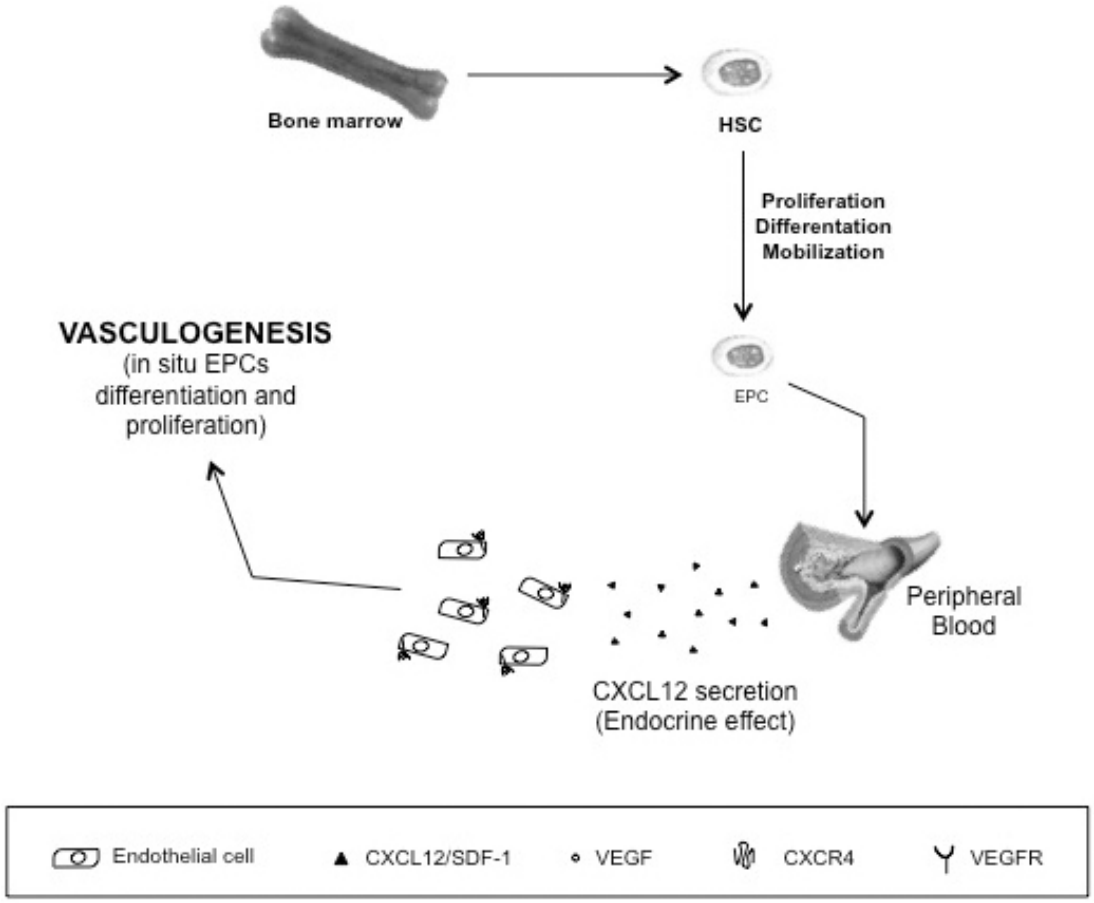

Fig. (2). Vasculogenesis. Schematic representation of CXCL12/CXCR4 axis and vasculogenesis mechanism. Recruitment of BMDC, BMDEPCs and EPCs from bone-marrow via CXCR4 pathway.

2 [31, 32]. In osteosarcoma, VEGF expression can be modulated by the transcription factor Yin Yang 1, while in others cancer angiogenesis occurs by different pathway [30, 33-36].

Metastasis Vascularisation: Preclinical Studies

Metastasis is a multiple step process. Initial events involve tumor cell detachment from the primary site, attachment to the vascular wall, and trans-endothelial migration into systemic circulation. *In vitro*, CXCL12 stimulates tumor cell motility via metalloproteinases (MMPs) and integrins activation [9]. Several *in vivo* studies confirmed this finding indicating that CXCL12 pathway is a key mediator of hepatic and bone metastasis hypothesizing that CXCL12 may be expressed by microenvironment [37-39]. *In vivo* models of breast cancer metastasis showed that CXCR4 directly recruits EPCs to metastatic site [28]. Consistent with these findings, metastasis and angiogenesis in pancreatic and lung tumor cells were promoted by CXCL12 and reduced by depletion of $CXCR4^+$ cells [40]. In addition, in osteosarcoma the inhibition of the CXCL12/CXCR4 axis reduced metastasis development as well as intra-metastatic and systemic vascularization in mouse models [41]. In contrast activation of CXCL12 pathway in response to certain chemotherapeutic drugs (e.g., Paclitaxel), anti-VEGF agents or irradiation, promoted angiogenesis, tumor growth and metastasis by recruiting BMDCs [42-44]. Taken together, these data demonstrate that in solid tumor, the blockade of CXCR4 pathway reduces but does not prevent metastasis formation, suggesting that CXCR7 may also be involved in this complex process. Recently, *in vivo* studies have been evaluating the use of new patented anti-CXCR7 drugs and combining anti-CXCR4 drugs with standard treatment and/or anti-VEGF therapy [45].

Tumor and Metastasis Vascularisation: Clinical Evidence

Microarray data from human biopsies or blood samples associated the CXCR4/CXCL12 axis with VEGF expression [20]. These pathways were correlated with poor outcome and tumor vascularisation in melanoma, osteosarcoma, and glioblastoma as well as pancreatic and colorectal carcinoma [46-50]. Immunohistochemistry assays showed a positive correlation between CXCR4 expression and microvessel density as a predictive value of lung metastasis development in osteosarcoma and melanoma patients [47, 51]. The increase of CXCL12 plasma levels, serum EPC number and BM $CD133^+$ cells reflected increased neoangiogenesis and tumor progression in early lymphatic leukaemia, as well as in pancreatic, rectal and renal carcinomas [52-55]. This latter finding reflects the clinical observation that high tissue expression of CXCL12 and CXCR4, is related to poor progno-

sis [51, 52, 56, 57]. Thus, CXCR4 inhibitors are being used to enhance chemotherapy efficacy in leukaemia.

The overlapping role of CXCR4 and VEGF in metastasis development is emerging from anti-VEGF therapy data, suggesting that the CXCL12 pathway is potentially involved in tumor resistance and recurrences [45]. In advanced hepatocarcinoma, soft tissue sarcoma and glioblastoma treatment VEGF blockade using pan-anti-VEGFR agents determined higher CXCL12 plasma levels, significantly associated with metastasis development [56-59]. These clinical data strongly suggest that CXCL12 pathway might be a potential therapeutic target to prevent tumor progression and improve neoadjuvant and adjuvant therapy in solid tumors.

Recent Anti-CXCR4 Patents in Cancer Therapy

The CXCL12/CXCR4 pathway could be an attractive target to modulate tumor angiogenesis, metastasis or chemoresistance. Several companies and academic centres have declared active programmes in this area and introduced new classes of drugs, Fig. (**3**).

CXCR4 ANTAGONISTS

Targeting CXCR4 with Antibodies

The introduction of neutralizing antibodies was the first approach used to block CXCR4 pathway in breast cancer tumor growth and metastasis (Table **1**) [60-68]. Monoclonal antibodies (Mabs) act as silent antagonists without any intrinsic activity on CXCR4 in absence of its ligand. This pharmacological feature is likely associated with less adverse side effects. A recent patent claimed Mabs directed against CXCR4 with anti-angiogenic activity: one group with an IC50 ranging 3-0.3nM (Table **1**, example 4) [63] and others with lower affinity (IC50 50nM and 44.8nM) (Table **1**, example 1,2) [60, 61]. In addition, in order to improve antibody affinity and potency and to further reduce side effects such as apoptosis of immune system cells, variable immunoglobulin domains and humanised antibodies have been patented. These molecules are currently in clinical trials for stem cell mobilisation in leukaemia treatment (Table **1**, example 7, 8) e.g., MDX1338 [66-68].

Small Synthetic Molecules: AMD3100 As Rapid Stem Cells Mobilizing Agent in Leukaemia Treatment

Emory University claimed a series of bicyclam compounds with general formula of JM3100 preventing the binding of monoclonal antibody 12G5 to CXCR4, inhibiting CXCL12 biological activity. Unfortunately, initially selected and patented as an anti-HIV drug, JM3100 showed a low antiviral effect. Subsequently, after further studies and the discovery of leucocytosis in patients treated with JM3100, Genzyme Corporation patented this bicyclam compound as AMD3100, which in 2008 received marketing approval (Mozobil™, Plerixaform) from the US Food and Drug Administration (FDA) for stem cell mobilisation in leukaemia [69, 70]. AMD3100 was extensively tested in haematological tumor cells and mice models, as pre-B acute lymphoblastic leukaemia (ALL), acute myelogenous leukaemia (AML) and myeloid leukaemia (ML), with a reduction of chemotactic homing and/or survival with lower toxicity (Table **2**, example 1) [71-78]. Later, AMD3100 was also used in the formulation of cyclic polyamine containing 9-32 ring, in combination therapy with granulocyte-colony stimulation factor (G-CSF) for haematologic disorders (Table **2**, example 1) [75, 76] or to enhance sensitivity to conventional multiple myeloma (MM) therapy [79].

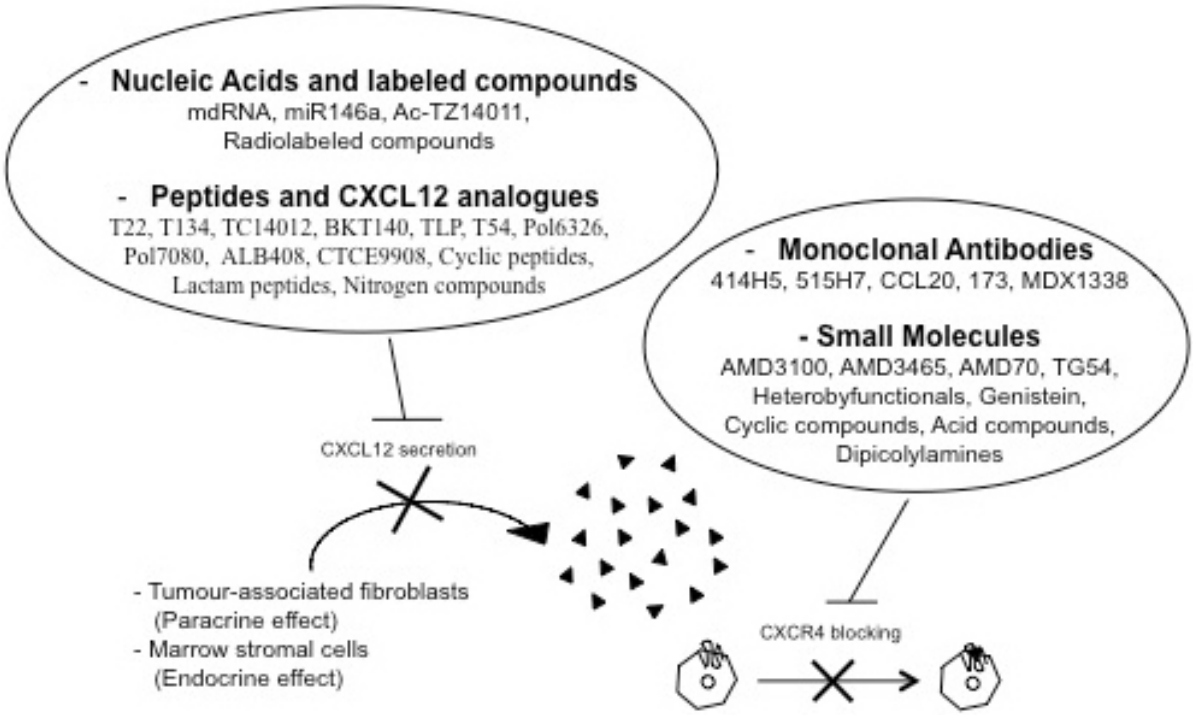

Fig. (3). Strategies for CXCL12/CXCR4 pathway inhibition. Strategies for CXCL12/CXCR4 pathway inhibition include both Peptides and CXCL12 ligand analogues that mimic CXCL12 secreted, and CXCR4 antagonists (Monoclonal antibody and Small molecules).

Table 1. Biological Activity of Recent CXCR4 Inhibitors Patented: Antibodies.

#	Antibodies	Company	Binding of Anti-CXCR4 mAb 12G5 to Cells (IC50)[1]	Stem/Tumor Cell Mobilisation and Angiogenesis	Tumor Growth and Metastasis	Ref.	WO
1	Monoclonal antibodies	Medarex, Inc.	50nM or less	YES	YES	[60]	WO2008060367
2	MAB 173	Pierre Fabre Medicament	44.8nM	ND	YES	[61]	WO2008142303
3	CCL20-specific antibodies	Hadasit Medical Research Services And Development Ltd.	ND	ND	ND	[62]	WO2009156994
4	Monoclonal antibodies	Eli Lilly and Company	0.3nM - 3.0nM	YES	YES	[63]	WO2009140124
5	414H5 and 515H7	Pierre Fabre Medicament	7.75nM	ND	YES	[64]	WO2010037831
6	Antibodies	Affitech Research As	ND	YES	YES	[65]	WO2011098762
7	Immunoglobulin single variable domains (MDX1338)	Ablynx Nv	ND	ND	YES	[66, 67]	WO2011083140 WO2011161266
8	Humanised antibodies	Pierre Fabre Medicament	ND	ND	YES	[68]	WO2011121040

[1]IC50: 50% Inhibitory concentration
ND: Not determine

Small Synthetic Molecules: AMD3100 in Solid Tumors

In vitro and *in vivo* experiments showed that AMD3100 was able to block chemotaxis, invasion and/or proliferation and tumor cell angiogenesis with a potency less than 50nM (Table **2**, example 1) [71, 72]. Preclinical models established that AMD3100 inhibits metastasis development in solid tumors such as breast cancer and glioblastoma as well as lung metastasis in melanoma (Table **2**, example 1) [73, 80-82]. Preclinical observations revealed that CXCL12 was generally overexpressed after standard therapeutic regimens in solid tumors, favouring recurrences and neo-angiogenesis [20]. For this reason, AMD3100 was extensively tested and patented as adjuvant in cancer treatment (Table **2**, example 1) [72, 74, 77]. Indeed, AMD3100 i.v. administration, in combination with chemotherapy, reduced tumor regrowth in mice model of Lewis lung and breast carcinomas [83]. However, the same study showed that AMD3100 had minor anti-tumor effects on established tumors [83]. In addition, several drawbacks have reduced its clinical application in solid tumors; AMD3100 showed opposite effects on neo-angiogenesis. On the one hand, AMD3100 antagonizes CXCL12 angiogenic activity, while on the other it stimulates EPC mobilisation from BM. These contrasting results suggest the need for further studies in order to extend its use in solid tumor treatment. Another AMD3100 pitfall is the lack of oral bioavailability due to its high overall positive charge.

AMD Related Compounds

AMD3465, a monocyclam, selected for its lower positive charge with respect to AMD3100, is still not orally bioavailable (Table **2**, example 2) [73]. However, it proved 10-fold more active as a CXCR4 antagonist compound compared to bicyclams. A pharmacokinetic study indicated that a single subcutaneous AMD3465 injection of 25mg/kg in mice led to rapid absorption and induced leukocytosis, with a peak cell mobilisation between 0.5 and 1.5 hrs after administration, demonstrating its therapeutic potential in cancer and EPC mobilisation. *In vitro*, AMD3465 inhibited stroma-mediated resistance to chemotherapy in leukaemic cells [84]. *In vivo* studies showed that this compound, alone or in combination with G-CSF, induced mobilisation of AML and EPCs into circulation, as well as enhancing the anti-leukaemic activity of chemotherapy and Sorafenib, prolonging animal survival [85]. In 2007, ChemoCentryx patented a new generation of monocyclam compounds with quinolozone as substituent group with anticancer activity (Table **2**, example 3) [86]. More recently, 33 claims patented Quinolone as TG-0054 drug in which quinolozone group was one substituent of AMD3465 for breast cancer treatment with an antiangiogenic activity [87], or in combination with a chemotherapeutic agent (Table **2**, example 3) [86, 87]. Among the AMD related compounds MSX-122 was the first anti-CXCR4 antagonist orally administered [88]. It is safer than cyclam compounds and contains two non-chelating pyrimidyl heterocycle substituents with low toxicity lacking metal-chelating moieties (Table **5**, NCT00591682) [88]. It has an IC 50 value less than 10nM and a half-life of 45 min. MSX-122 is currently in Phase I trials to test tolerance and side effects in healthy patients.

Table 2. Biological Activity of Recent CXCR4 Inhibitors Patented: Small Molecules.

#	Small Molecules	Company	Binding of AntiCXCR4 (IC50) mAb 12G5 to Cells (IC50)[1]	CXCL12 Induced Effects *in vitro*[2]	Cell Proliferation (CC50)[3]	Stem/Tumor cell Mobilisation and Angiogenesis	Tumor/ Growth and Metastasis	Ref.	WO
1	AMD 3100	Genzyme Corporation	37nM	1-5μM	> 300μM	YES	YES	[71]	WO2004087068
						"	"	[72]	WO2006074428
						"	"	[73]	WO2007022523
						"	"	[74]	WO2008008854
						"	"	[75]	WO2008017025
						"	"	[75]	WO2008019371
						"	"	[76]	WO2010088398
						"	"	[77]	WO2011014863
						"	"	[78]	WO2011123903
2	AMD3465	Genzyme Corporation	1nM	1μM	> 300μM	YES	YES	[73]	WO2007022523
3	Quinolone TG-0054	ChemoCentryx, Inc.	ND	ND	ND	YES	YES	[86]	WO2007059108
4	Genistein	Axcentua Pharmaceutucal Ab	ND	ND	ND	YES	YES	[90]	WO2010068861
5	Chalcones 4	Sigma	ND	ND	ND	ND	YES	[118]	WO2011151789
6	Heterobifunctional Inhibitors	Glycomimetics, Inc.	8.25μM	ND	ND	YES	YES	[123]	WO2010126888
7	CCX-2066 (Anti CXCR7)	Metastatix Inc.	100 - 500nM	ND	ND	YES	ND	[124]	WO2010054006

[1]IC50: 50% Inhibitory concentration
[2]Inhibition of chemotaxis and/or signaling
[3]CC50: 50% Cytostatic concentration
ND: Not determine

Other Small Synthetic Compounds

WZ811 is an orally available compound designed and selected for its high affinity to CXCR4. It showed a potency subnanomolar (EC50 = 0.3nM) in affinity binding assays and inhibited the angiogenic activity of MDA-MB-231 breast carcinoma cells [89]. Genistein (Table **2**, example 4) [90], a dietary phytoestrogen belonging to the class of flavonoids, in addition to its inherent anti-tumor effects, has shown to increase the effects of several chemotherapeutic agents both *in vitro* and *in vivo* in animal models by blocking EPC recruitment via CXCR4 pathway (Table **2**, example 4) [90].

Peptides and Analogues

The screening of natural molecules generally, less toxic than synthetic drugs has selected polyphemusin from the haemocytes of American horseshoe crabs as being able to block CXCR4 signalling pathway. Peptides, analogues of this molecule, showed higher antiangiogenic activity than synthetic compounds (Table **3**) [91]. T22 (Table **3**, example 1) [92, 93] was the first peptide patented for antitumor activity with IC50 of 48nM and CC50 of 13μM. Subsequently, the length of this molecule was reduced to 14mer in T134 (Table **3**, example 2) [93, 94] and TC14012 peptides (Table **3**, example 3) [71, 95] with low toxicity (CC50 greater than 800μM). Both peptides were able to inhibit *in vivo* leukaemic cell dissemination (Table **3**, example 2,3) [71,95], metastasis from head and neck cancer, breast cancer, and osteosarcoma cells, where T22 administration reduced intrametastatic vessel formation and systemic neo-angiogenesis [41, 96, 97]. However, the major issue in the use of these peptides was serum stability, which several derivatives were unable to improve [94]. Otherwise, the introduction of acetylation in the amino-terminus region improved both antitumor activity and bio-stability. TN14300 or BKT140 (Table **3**, example 4) [93], selected for more promising anti-tumor effects, binds CXCR4 at a concentration of 0.1nM, reduced intra-metastatic vascularisation of pancreatic cancer cells and breast carcinoma, as well as head and neck tumor growth and metastasis [96-99].

Table 3. Biological Activity of Recent CXCR4 Inhibitors Patented: Peptides.

#	Peptides	Company	Binding of Anti-CXCR4 (IC50)[1]	CXCL12 Induced Effects *in vitro*[2]	Cell Proliferation (CC50)[3]	Stem/Tumor Cell Mobilisation and Angiogenesis	Tumor Growth Metastasis	Ref.	WO
1	T22	Human Genome Sciences, Inc.	48nM	3μM	13μM	ND	YES	[93]	WO2001079444
2	T134	Human Genome Sciences, Inc.	4nM	0.5μM	> 100μM	YES	YES	[93]	WO201079480
3	TC14012	Emory University	1nM	40μM	> 800μM	YES	ND	[71]	WO2004087068
4	TN14003 or BKT140	Emory University	ND	0.1μM	410μM	YES	YES	[93]	WO2001079480
5	Nitrogen compounds	Kureha Chemical Industry Company	ND	ND	ND	YES	YES	[100]	WO2002094261
6	Lactam-cyclized peptide	Eli Lilly and Company	0.6 - 6nM	ND	ND	YES	YES	[101]	WO2008150689
7	Template-fixed beta-hairpin peptidomimetics (pol6326- pol7080)	Polyphor Ag Polyphor Ltd.	0.7 – 10μM 16.6μM	ND	ND	YES	YES	[102] [105]	WO2010060479 WO2008104090
8	Beta-hairpin peptidomimetics	Polyphor Ag	1.8 - 95.5μM	ND	ND	YES	YES	[103] [104]	WO2011066869 WO2010127704
9	ALB-408	Pharis Biotec Gmbh	10 - 20μM	ND	ND	YES	YES	[106]	WO2009004054
10	TLP (Idrophilic linker, peptides)	Anchor Therapeutics, Inc.	1.30 - 2.66μM	ND	ND	ND	ND	[107] [108] [110]	WO2010037042 WO2011106703 WO2010092571
11	T54 Oncofoetal glycoprotein	Cancer Research Technology Limited	ND	ND	ND	ND	ND	[109]	WO2011048369
12	Cyclic peptides	National Research Council	ND	ND	ND	YES	YES	[111]	WO201109257
13	CTCE-9908	Dana-Farber Cancer Institute	ND	40μM	ND	YES	YES	[119]	WO2006089141

[1]IC50: 50% Inhibitory concentration
[2]Inhibition of chemotaxis and/or signalling
[3]CC50: 50% Cytostatic concentration
ND: Not determine

Peptidomimetics and Derivative Peptides

Peptidomimetic molecules were developed to prolong peptide half-life *in vivo* and enhance CXCR4-antagonizing properties (Table **3** example 5-12) [100-111]. Peptidic backbone with nitrogenous functionality from Kureha Pharmaceuticals consists of a general formula containing arginine or arginine analogues on one or both ends of the molecule (Table **3**, example 5) [100]. All EC50 values were under micromolar level with no notable side effects up to dosing levels of 15mg/Kg twice daily for 4 days [100]. In 2008, backbone peptides were modified with beta-lactam rings (Table **3**, example 6) [101], polyphor (Table **3**, example 7) [102] or beta-harpin cyclic groups (Table **3**, example 8) [103], a cyclic structure attached to NO group as terminal arginine mimics (Table **3**, example 7,8) [104, 105], or a beta-harpin linked to linear peptide (Table **3**, example 9) [106]. These new molecules exhibited greater potency compared to linear peptides with IC50 values ranging from 0.7μM to 10μM and showed antiangiogenic and stem cell mobilisation properties as well as being indicated as diagnostic tool. Other peptides with general formula TLP (hydrophilic linker peptides or glycol

peptide were patented in 2011 (Table **3**, example 10,12) [107-109], as allosteric modulators (e.g., positive and negative allosteric modulators, and allosteric agonists) of CXCR4 able to prevent angiogenic activity of tumor cells (Table **3**, example 10) [110]. Specifically, the invention provides compositions and methods useful for promoting HSC and EPC mobilisation and transplantation. The Italian National Research Council claimed the identification of new peptides and peptidomimetics, which bind and form complexes with CXCR4 (Table **3**, example 12) [111]. The invention relates to the use of these peptides for the treatment, prevention and diagnosis of neoplasia and metastasis, as well as stem cell mobilisation in autologous transplantation. Unfortunately, for most of these molecules data regarding antiangiogenic activity are not available, and when it is reported, it is difficult to compare their potency.

NUCLEIC ACID COMPOUNDS ACTING AS INTRACELLULAR CXCR4 SILENCER

Meroduplex ribonucleic acid (mdRNA) molecules comprise a first strand of 15 to 40 nucleotides in length able to decrease or silence CXC (e.g., CXCL12, CXCR4, or CXCR7) gene expression in preclinical studies with antiangiogenic activity in tumor cells (Table **4**, example 1) [112]. MicroRNAs (miRNAs) have also proved to be a valid alternative strategy for cancer treatment [113]. miRNA 146a (Table **4**, example 2) [114] tested in breast cancer was recently patented as CXCR4 antagomir by the Italian Ministry of Health.

Labelled CXCR4 Binders as Biomarker

The development of biomarkers to monitor therapeutic efficacy is a critical issue in tumor treatment. Ac-TZ14011 (Table **4**, example 3) [115] was the first labelled fluorophore derived from T140 peptide, used to monitor $CXCR4^+$ cell *in vivo* by fluorescence microscopy. Moreover, labelled CXCR4 is currently used for PET scan analysis of CXCR4 positive tumors in mice model and as marker of therapeutic efficacy (Table **4**, example 3) [115]. Emory University recently patented a radiolabelled CXCR4 antagonist, which may be used to image CXCR4 expression by PET, SPECT, MRS, MRI and optical imaging compositions in all pathological conditions associated with the expression of CXCR4 including cancer and metastasis (Table **4**, example 4) [116].

INHIBITORS OF CXCL12

Nucleic Acids

Nox-A12, from Noxxon Pharma, has different mechanisms of action. Nox-A12 is an aptamer nucleic acid able to bind CXCL12 preventing its action on receptors. This compound is currently in trials in combination with standard therapy for treatment of chronic lymphatic leukaemia (Table **4**, example 5) [117].

Small Synthetic Compounds

Another class of compounds includes Pyridinyl compound -like molecules, 3-(2-methyl-1H- indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (MIPP). Among them Chalcone 4 from Sigma (C7870) is able to inhibit the binding of CXCL12 to CXCR4 and CXCR7 with high chemokine binding affinity and antiangiogenic activity (Table **2**, example 5) [118].

Peptides and Peptidomimetics

The group of CXCL12 analogue peptides is a further class of CXCR4/CXCL12 signalling pathway inhibitors. The most promising is CTCE-9908 (Table **3**, example 13) [119], consisting of a dimer of CXCL12 (Chemokine Therapeutics Corporation, Vancouver, BC), which acts as a competitive inhibitor, as demonstrated by radioligand binding assays. CTCE-9908 inhibited tumor growth and metastasis in osteosarcoma, melanoma, prostate and breast cancer mouse models [120-122]. For instance, a recent study evaluating the efficacy of this CXCR4 antagonist, alone or in combination with paclitaxel, showed that CTCE-9908 single therapy was able to reduce the size and intra-metastatic vascularisation in different organs [120-122].

Table 4. Biological Activity of Recent CXCR4 Inhibitors Patented: Nucleic acid and Labelled Compound.

#	Nucleic Acids and Labelled Compounds	Company	Binding of Anti-CXCR4 (IC50)[1]	Stem/Tumor Cell Mobilisation and Angiogenesis	Tumor Growth and Metastasis	Ref.	WO
1	Meroduplex ribonucleic acid molecules (MDRNA)	Mdrna, Inc.	ND	ND	ND	[118]	WO2008109520
2	miRNA 146a	Institute of Health	ND	YES	YES	[114]	WO2009100955
3	Ac-TZ14011	Biokine Therapeutics Ltd.	1.2 - 7.9 nM	ND	YES	[115]	WO200220561
4	Radiolabelled	Emory University	ND	ND	YES	[116]	WO2011094389
5	Nox-A12 Aptamer	Noxxon Pharma AG	ND	YES	YES	[117]	WO2009019007

[1]IC50: 50% Inhibitory concentration
ND: Not determine

Antagonists of Heterodimers CXCR4 and CXCR7

New drugs with hetero-bifunctional activity have been selected to block CXCL12 effects on both CXCR4 and CCR7, often present as heterodimer in solid tumors.

Adelaide Research & Innovation Pty Ltd. patented a heteromultimer of AMD3100 (Table **2**, example 1) [78] able to bind both CXCR4 and CXCR7, as well as a combination of AMD3100 and MMP9 inhibitors (Table **2**, example 1) [77] as preventive methods for metastasis treatment. The addition of glycogen group linked to non-cyclic group produces a compound (Table **2**, example 6) [123] binding both CXCR4 and CXCR7, with a formulation for any appropriate manner of administration and with prolonged release. In addition, CCX-2066, a small synthetic compound with high affinity to CXCR7 tested in preclinical studies with promising results (Table **2**, example 7) [124].

Drug Limitations

The biological activities of the most extensively studied compounds summarised in Tables **1**-**5** showed that small molecules were initially favoured as antagonists for clinical use, but their accessibility to the target (size and systemic route of administration) as well as broad expression of CXCR4, often led to unwanted side effects. Small peptides are being increasingly considered as therapeutic agents with high selectivity, easy to engineer, locally administer (e.g., intra-tumoral injection) and with a long half-life that prolongs intervals between treatments. However, the poor selectivity of these compounds is often a cause of failure in preclinical phase. Limitations in bringing these agents to the market are linked to the complexity of human tumor environment, in which chemokines may play several roles compared to murine models. Moreover, preclinical studies agreed with the following findings: 1) anti-CXCR4 therapy alone is not sufficient to inhibit tumor growth except for certain solid tumors; 2) anti-CXCR4 agents had minor anti-tumor effects on established tumors and did not prevent metastasis development; 3) the efficacy of more effective drugs should be dosed in combination with anti-VEGF agents, chemotherapy, or irradiation; 4) drugs designed to bind and block CXCR4 may not be sufficient to block the effects of CXCL12, which may also bind to CXCR7 as homodimer or heterodimer on cancer or stromal cells. Hetero-bifunctional inhibitor molecules with both binding activity have begun to be tested, but data are not yet available. In addition, CXCR7 signal transduction pathway as well as the role of CXCR4 and CXCR7 in the immune system have still not been completely elucidated.

Clinical Applications

Main clinical studies involving CXCR4 antagonists are currently investigating their use as stem cell mobilisers in leukaemia patients. At time of writing only MSX-122 (Metastatix) is in Phase I trial for solid tumor treatment (breast and prostate cancer) (Table **5**).

AMD3100, approved by FDA in 2008 with the name of Plerixafor, was used as adjuvant treatment in leukaemia patients. AMD3100 can promote migration of haematopoietic stem cells and/or EPC from BM into circulation, facilitate recovery after immunosuppression and reduce retention of AML cells in BM, responsible for chemoresistance [125, 126]. Currently Phase III trials are evaluating the ability of AMD3100 to mobilize stem cells alone or in combination with G-CSF for treatment of non-Hodgkin's lymphoma [127]. Recent patent applications expand the use of AMD-3100 in combination with CXCR2 antagonist GRO β protein or in combination with chemotherapy (NCT00512252; NCT01027923; NCT01220375) in MM an non-Hodgkin's patients [127, 128]. Other studies are combining AMD3100 with or without AC220, a tyrosine kinase inhibitor, (NCT01236144; NCT01236144) or with C-CSF (NCT0073-3824). However, to improve clinical benefit and reduce dose intensity and toxicity, other molecules such as the antibody MDX-1338/BMS-936564 (NCT01120457), or the peptide BKT140, oral administered, (4F-benzoyl-TN14003) are currently in clinical trials to test their safety and efficacy in AML patients [129]. In addition, in a study recently completed but not yet published, BKT140 was tested in combination with rapamycine in MM patients (NCT01010880). These observations have led to the possibility of using these molecules as a therapeutic adjuvant approach in several malignancies, such as leukaemia, NHL and MM. The aptamer Nox-A12 (from Noxxon Pharma AG) is a different class of drugs in multi-centre Phase IIa clinical trials to evaluate safety and efficacy in combination with a background therapy in patients with chronic lymphocytic leukaemia (CLL) (NCT01486797). Finally, a randomised double-blind study in healthy volunteers is testing the plasma pharmacokinetic profile and pharmacodynamic effects of TG-0054, a new generation quinolone antibiotic (NCT01018979).

MSX-122 (Metastatix), orally administered, is currently in Phase I trials for solid metastatic breast tumors (NCT00591682) [88, 130, 131]. Data available indicates that this trial is suspended, and unfortunately no results have been published. However, there is no doubt concerning CXCL12 proangiogenic role in cancer, and the development of specific biomarkers remains a critical issue for anti-CXCR4 as an effective therapy.

CURRENT & FUTURE DEVELOPMENTS

In multiple tumors CXCL12 is a common pathway to grow and escape therapy, but its systemic effects are critical in solid tumor treatment, which is longer than haematologic tumor therapy, thus increasing side effects. CXCR4 antagonists may not only induce mobilisation of haematological or metastatic cancer cells from their protective niche in BM, but, at the same time, may also mobilize normal stem cells, leading to increased toxicity of chemotherapy. Furthermore, in the tumor microenvironment CXCR4 antagonists may inhibit CXCL12 angiogenic activity, but may also recruit EPCs, leading to enhanced angiogenesis, tumor re-growth and increased risk of metastasis.

Recent preclinical studies showed that combination of conventional therapies with CXCR4 inhibitors will be a challenge in patients with solid and haematological tumors. However, another critical point for the success of a novel combination therapy in solid tumor is the lack of approved biomarkers to monitor angiogenesis in order to determine the

Table 5. CXCR4 Antagonists Clinical Trials Ongoing.

ClinicalTrials.gov Identifier	Structure	Indication	Intervention (Drug)	Phase	Study
NCT00322127	Bicyclam	Stem cell mobilization	AMD3100	I	An evaluation of safety and efficacy of escalating doses of AMD3100 to mobilize $CD34^+$ cells in healthy volunteers
NCT00075335	Bicyclam	Healthy; blood component removal	AMD3100	II	AMD3100 to mobilize stem cells for donation
NCT01058993	Bicyclam	Neutropenia	AMD3100	I	AMD 3100 for treatment of myelokathexis
NCT01068301	Bicyclam	Acute lymphoblastic leukemia; acute myeloid leukemia; chronic myeloid leukemia; myelodysplastic syndrome; Non-Hodgkin's lymphoma	AMD3100	I	A pediatric study of a Plerixafor containing regimen in second allogeneic stem cell transplantation
NCT01236144	Bicyclam	High risk myelodysplastic syndrome	AMD3100; AC220 with chemo-therapy	I - II	A trial to establish the feasibility of combining either the tyrosine kinase inhibitor AC220 or the CXCR4 Inhibitor Plerixafor with chemotherapy in older patients with AML and high risk myelodysplastic syndrome
NCT01220375	Bicyclam	Stem cell mobilization In myeloma	AMD3100; Chemotherapy	II	PAV-trial:Plerixafor and chemotherapy with Vinorelbine in patients with myeloma
NCT01027923	Bicyclam	Acute myeloid leukemia	AMD3100; Chemotherapy	I	Plerixafor with Mitoxantrone Etoposide and Cytarabine for AML
NCT00512252	Bicyclam	Acute myeloid leukemia	AMD3100; Chemotherapy	I - II	AMD3100 plus Mitoxantrone, Etoposide and Cytarabine in acute AML
NCT00906945	Bicyclam	Acute myeloid leukemia	G-CSF; AMD3100; Chemotherapy	I - II	Chemosensitization+Plerixafor plus G-CSF in AML
NCT00733824	Bicyclam	Lymphoma, Non-Hodgkin, Hodgkin disease	G-CSF; AMD3100; Apheresis	I - II	Intravenous AMD3100 for collection of autologous peripheral blood stem cells in patients with lymphoma
NCT01120457	Bicyclam	Acute myeloid leukemia	MDX-1338/BMS-936564	II	MDX1338 in human study to determine the safety tolerability and effectiveness in subject with AML
NCT01010880	Modified peptide	Multiple myeloma	BKT140	Completed	Safety study of a chemokine receptor (CXCR4) antagonist in MM patients
NCT01413568	Polyphemusin II derived	Leukemia Lymphoma Multiple myeloma	POL6326	II	Determine the safety and toxicity of POL6326 as a mobilization agent
NCT01018979	Quinolone	Multiple myeloma non-Hodgkin lymphoma Hodgkin disease	TG-0054	II	Multi-center study to evaluate the safety, pharmacokinetics, and hematopoietic stem cell mobilization in patients with MM, NHL or Hodgkin disease
NCT00591682	Synthetic compoud	Solid tumors	MSX-122	I	MSX-122 administered orally in patients with refractory metastatic or locally advanced solid tumors
NCT01486797	Aptamer	Lymphocytic leukemia	NOX-A12	II	Chronic lymphocytic leukemia Nox-A12 + conventional therapy

degree and duration of target 'knockdown' and to dose toxicity of novel targeted therapies. Moreover, future research should gain a more in-depth understanding of CXCL12/CXCR4/CXCR7 pathway to translate CXCL12, CXCR4 or CXCR7 inhibitors into clinical practice.

Finally, it is unclear what long-term effects CXCR4 antagonism may have on innate and adaptive immunity where CXCL12 guides immune surveillance against tumors. This issue is difficult to study in current nude mice models routinely used to test these antagonists. Only long-term analysis of current clinical studies and/or novel mice models may provide key insights into CXCR4 antagonist application.

ABBREVIATIONS

ALL	=	Acute lymphoblastic leukaemia
AML	=	Acute myeloid leukaemia
BM	=	Bone marrow
BMPC	=	Bone marrow derived progenitor cells
CAF	=	Carcinomas associated fibroblast
CKR	=	Chemokine receptor
CLL	=	Chronic lymphoblastic leukaemia
CXCL12	=	Chemokine ligand 12
CXCR4	=	Chemokines receptor 4
EC	=	Endothelial cells
EPC	=	Endothelial progenitor cells
G-CSF	=	Granulocyte- colony stimulation factor
HIF-1	=	Hypoxia-inducible factor-1
HPC	=	Hematopoietic progenitor cells
HSC	=	Haematopoietic stem cells
MM	=	Multiple myeloma
NHL	=	non-Hodgkin's Lymphoma
PlGF	=	Placenta growth factor
SDF-1	=	Stromal-derived factor 1
VEGF	=	Vascular endothelial growth factor
VEGFR1	=	Vascular endothelial growth factor receptor I

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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