G-Coupled Protein Receptors and Breast Cancer Progression: Potential Drug Targets

Marcelo Taborga[ζ], Kelly E. Corcoran[ζ], Neil Fernandes[#], Shakti H. Ramkissoon[ζ], Pranela Rameshwar[ξ,*]

[ξ]*New Jersey Medical School-UMDNJ, Department of Medicine, Division of Hematology/Oncology;* [#]*Department of Medicine, UMDNJ-New Jersey Medical School, Newark, NJ;* [ζ]*Graduate School of Biomedical Sciences, UMDNJ, USA*

Abstract: Breast cancer remains a leading cause of death despite early screening and advances in medicine. Bone marrow metastasis often complicates the clinical picture by requiring more aggressive treatment and worsening long-term prognoses. Recent therapeutic targeting of hormonal receptors such as human epidermal growth factor receptor 2 and estrogen receptor has shown limited success in treating localized disease for those patients whose cancer cells are responsive. Although traditional approaches such as chemotherapy have demonstrated many successes, these agents fail to target quiescent cancer stem cells, which might have entered the bone marrow where they might be responsible for the quiescence population. Following years of clinical remission, these dormant cells could lead to secondary cancer resurgence. To date, little progress has been made in the development of targeted treatments for receptor negative and metastatic disease. In this review, we discuss the role of G-protein coupled receptors, including neurokinin-1, neurokinin-2 and chemokine receptor 4, as novel targets in the treatment of breast cancer.

Key Words: CXCR-4, tachykinins, metastasis, chemokines, cancer, hematopoiesis.

INTRODUCTION

The detection of breast cancer (BC) during the early phase of the disease has improved over the last few years. This is partly due to advocacy for self-screening, education, advanced imaging and improved diagnostic modalities. However, despite these and other advances, BC remains a leading cause of death among women in the United States [1]. BC preferentially metastasizes and invades the bone marrow by utilizing existing hematopoietic stem cell homing mechanisms, allowing them to stably integrate within the bone marrow microenvironment [2, 3]. The establishment of BC cells in the bone marrow is a clinical problem, since the experimental evidence indicates that the integrated cancer cells can evade detection by transitioning into a quiescent state with respect to cell cycle. This quiescence, in turn, provides the BC cells with protection from chemotherapeutic agents, which require proliferating cells for effective clearance of the cancer cells. Another limitation to effective therapy is based on the biology of the finite number of resident hematopoietic stem cells [4]. At high dose of chemotherapy, the stem cells could be subjected to its toxic effects. This therapeutic dilemma is challenging to both basic scientists and clinicians. However, the most critical issue is the poor prognosis from metastatic disease. Although currently available medications such as herceptin and tamoxifen are useful for targeting BC at the primary site, bone marrow invasion remains an enduring problem.

As the clinical and therapeutic outlook seem to be currently tenuous, there is considerable need for the development of novel drugs to target both primary and secondary BC. The G-protein coupled receptors (GPCRs) are a large group of bioactive molecules that show promise as potential targets for BC therapeutics. These receptors have many functions including cell proliferation and angiogenesis [5]. Examples of G-protein coupled receptors that are relevant to BC are those that interact with neuropeptides and chemokines [6]. Dysregulation of GPCR signaling has been implicated in cancer and other diseases. Therefore, it is not surprising that over half of current therapeutic targets are aimed at GPCRs or their signaling pathways [7]. While GPCRs could be potential targets for BC, this strategy has proven to be much more complex since, to date; there is no single 'magic bullet' among the GPCRs.

OVERVIEW OF G-PROTEIN COUPLED RECEPTORS (GPCRS)

GPCRs signal through multiple intracellular pathways and are involved in the development of BC and perhaps other endocrine-related cancers [8]. Here we discuss two subfamilies of GPCRs, CXCR4 and NK receptors with respect to their normal biology and potential roles as targets in treating local and metastatic disease. GPCRs, or seven transmembrane receptors, encompass a vast superfamily of cell surface proteins involved in signal transduction. To date, there are >800 identified GPCRs that signal by activating coupled G-proteins proportionately to agonist concentrations [9]. Their biological functions are numerous in both normal homeostasis as well as in diseased states.

In the absence of ligand binding, G-proteins and transmembrane receptors exist in a bound, inactive state. Inactive G-proteins exist as a heterotrimeric unit composed of three subunits: $G\alpha$(s, i, q, o, t), $G\beta$, $G\gamma$, where binding of a GDP (guanosine diphosphate) molecule to the $G\alpha$ subunit is char-

*Address correspondence to this author at the UMDNJ-New Jersey Medical School, MSB, Rm. E-579, 185 South Orange Ave, Newark, NJ 07103, USA; Tel: (973) 972 0625; Fax: (973) 972 8854;
E-mail: rameshwa@umdnj.edu

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acteristic of the inactive state. Upon ligand binding to the receptor, the exchange of GDP for GTP (guanosine triphosphate), in the Gα subunit, is typically followed by dissociation of Gα from the Gβ/γ subunit. Uncoupling of the G-protein subunits occurs concurrently with their detachment from the receptor, and both the Gα and Gβ/γ subunits are capable of activating downstream targets. The specific targets of Gα-GTP depend on the specific Gα subtype, but commonly include adenylyl cyclase (AC), depicted in Fig. (**1**), and phospholipase C (PLC). The Gβ/γ subunit, on the other hand, typically targets phosphatidylinositol-3-kinase (PI3K) and PLCβ for activation.

The GPCRs that is discussed in this brief review are two members of the neurokinin (NK) receptor family (NK1 and NK2) and the chemokine receptor 4 (CXCR4). Each of these receptors has been linked to the development of BC. NK1 and NK2 are implicated in the proliferation of BC cells, and a truncated form of NK1 has been linked to transformation of non-tumorigenic mammary epithelial cells [10]. Additionally, the CXCR4 receptor has been identified as a major participant in cancer metastasis [11, 12]. Together, NK1, NK2 and CXCR4 receptors represent a set of molecular targets within a common family that could be ideal molecular targets, either single, or as adjuvant to other conventional treatment modalities.

STROMAL DERIVED FACTOR-1 (SDF-1)/CXCR-4

Chemokines are small molecules, 6-14 kDa, originally characterized by their chemotactic effects. They exist as either secreted or membrane-bound forms and are classified into four main categories based on structure [13]. SDF-1, also known as CXCL12, is a highly conserved, low molecular weight (8-10 kDa) chemokine that is chemotactic [14]. Two variants, SDF-1α and SDF-1β, are derived from alternatively spliced transcripts and differ by the addition of four amino acids (RLKM) at the C-terminus of the β variant [15].

CXCR4 is a 7-transmembrane, G-protein coupled receptor for both forms of SDF-1 [14]. A recent report has identified syndecan 4 as an alternate binding partner for CXCR4 [16]. In addition to BC cells, CXCR4 is also expressed on hematopoietic cells and endothelial cells [17]. CXCR4 exerts its actions through both tyrosine kinase and $G\alpha_i$, leading to the mobilization of intracellular calcium, activation of focal adhesion kinase proteins and the activation of transcription factors such as NFκB [17, 18]. SDF-1-CXCR4 interaction is involved in cell adhesion, chemotaxis, migration, cell proliferation, cell survival and development within various organs [14].

In the bone marrow microenvironment, SDF-1α is produced by stromal cells and released into the surrounding extracellular matrix where it is retained by surface proteoglycans creating a natural gradient [19]. SDF-1α gradient is partly responsible for the localization of resident hematopoietic stem cells where SDF-1α is at its highest concentration, thus establishing a niche close to endosteum of the bone marrow cavity. It is this region that the BC cells appear to integrate, with the SDF-1α controlling how the cells integrate to avoid detection [20]. This same endosteal region is also ideal for cells that are in need of protection from reactive oxygen species since this area has low oxygen concentration [21]. Disruption of the SDF-1 gradient releases

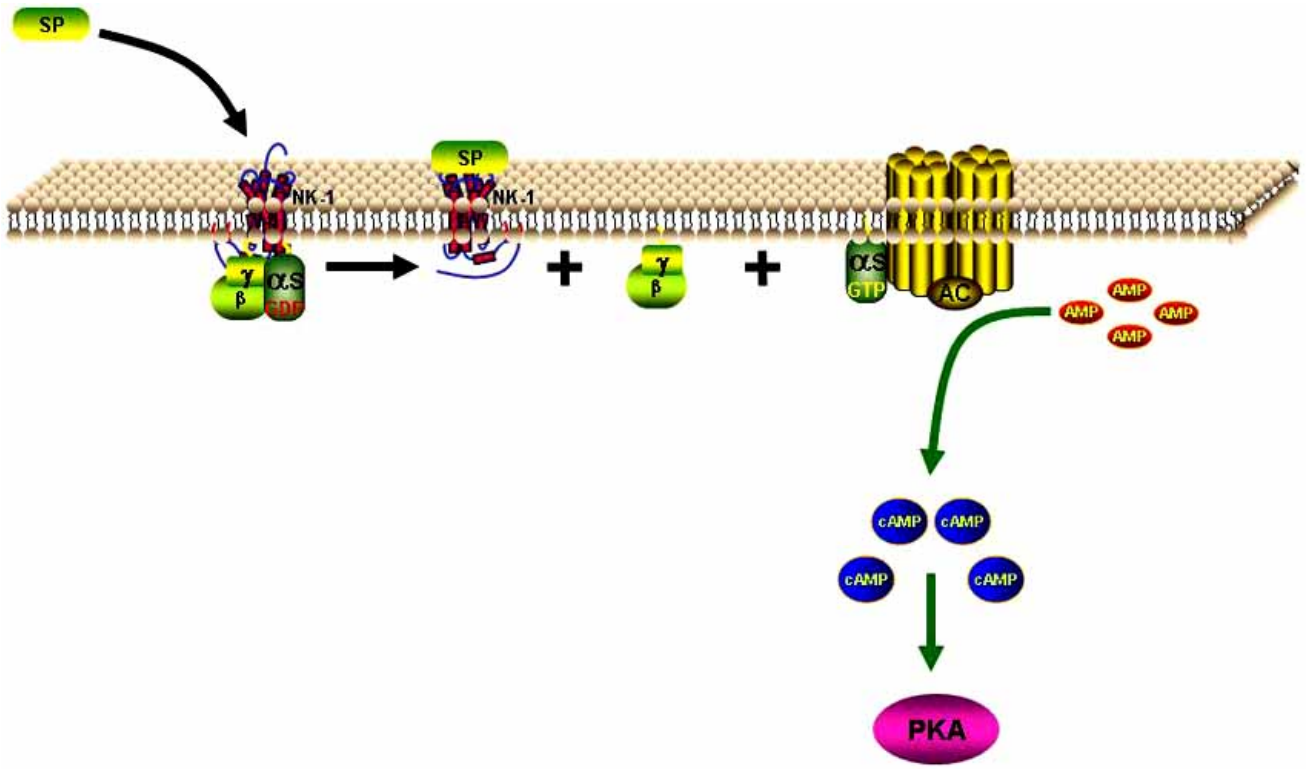

Fig. (1). Activation of G-proteins by ligand binding to a transmembrane receptor. Here, the NK-1 transmembrane receptor is activated by SP. NK-1 activation stimulates cAMP to phosphorylate PKA.

CXCR4-expressing cells, such as hematopoietic stem cells thereby releasing them to home out of the bone marrow and into the peripheral circulation and organs [22]. BC cells that might be close to the endosteum could be similarly released to cause tertiary metastasis. Agents capable of uncoupling SDF-1/CXCR4 include chemotherapy, cytokines, matrix metalloproteases and neutral endopeptidase, CD26 [23].

CXCR4 AS POTENTIAL DRUG TARGET

CXCR4 antagonists, AMD3100 and AMD3465, are potential agents that mobilize CXCR4+ cells from the bone marrow into the periphery [24]. AMD3100 is a symmetric bicyclam, non-peptide molecule that interacts with the 7-transmembrane (TM) spanning CXCR4 receptor at single residues in TM-IV, VI and VII [25]. AMD3100 has been shown to have anti HIV-1 activity, in particular the strains that require cell entry through the CXCR4 receptor [26]. The course taken to develop AMD3100 is of interest as it shows how a contaminant by a compound provide insight into an antagonist that might have great clinical value [27]. The original prepartion was a contaminant of a cyclam with anti-HIV activity [26]. Once the purified preparation could not replicate the anti-HIV properties, this led medicinal chemists to understand the contaminating compound and then use this informatio to synthesize several others, which subsequently lead to the bicyclam AMD3100 [27]. During clinical trial with AMD3100 for HIV-1, the subjects showed high white blood cell counts. This led to the discovery that AMD3100 could mobilize hematopoietic stem cells from the bone marrow [27]. Subsequent research studies proved AMD3100 to be highly specific for CXCR4 thereby giving this compound potential for other clinical application, including those with inflammation as underlying causes [28]. Due to the poor bioavailability of AMD300, a modified molecule was generated, which is a monomacorcyclin cyclam, designated, AMD3465 [29]. In addition, this modified compound shows better efficacy as an anti-HIV-1 agent [30].

CXCR4 antagonists could be potential agents for treating early, 'pre-metatstatic' BC as the antagonist could disrupt interaction close to the endosteum. However, such gains would come at the expense of hematopoietic stem cell mobilization. Despite this complication, these agents represent a novel therapeutic strategy for prophylaxis approach for BC and other endocrine cancers that metastasize to the bone marrow. Further studies are needed to evaluate correct dosing for prophylaxis purposes without untowards effects of bone marrow functions, including hematopoietic stem cell mobilization. Perhaps a low dose can be administered systemically to provide the 'anti-metastatic' effects without significant hematopoietic alterations. Another strategy would involve local administration of these agents, in hope to inhibit BC migration from the site of primary tumor.

A more radical use of these agents would be for the treatment of established bone marrow metastases. As SDF-1/CXCR4 interaction is responsible for maintaining hematopoietic stem cells in the bone marrow, it also serves a similar role for BC. As such, these antagonists could be used to mobilize BC cells from the bone marrow into the periphery where they can be targeted for destruction. Given the seemingly common mechanism in the mobilization of BC cells and hematopoietic stem cells, any treatment strategy aimed at BC must be highly specific in order to limit the loss of the finite number of hematopoietic stem cells.

OVERVIEW OF TACHYKININS AND THEIR RECEPTORS

The mammalian tachykinins are a family of neuropeptides that share common C-termini, Phe-X-Gly-Leu-Met-NH2, where X could be Phe, Val, or Tyr [31]. This conserved sequence is essential for both receptor interaction and subtype specificity [31, 32]. Mammals express several tachykinin peptides such as substance P (SP), neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK), neuropeptide-γ(NPγ) and hemokinin-1 (HK1). Three distinct genes encode mammalian tachykinins, preprotachykinin-A (*PPT-A*), *PPT-B* and *PPT-C* [31-33]. *PPT-A* is a single copy gene with seven highly conserved exons [31]. Fig. (**2**) depicts the alternative splicing of *PPT-A* mRNA yields four different transcripts (α, β, δ and γ) that encode for SP, NKA, NPK, and NPγ [32, 33].

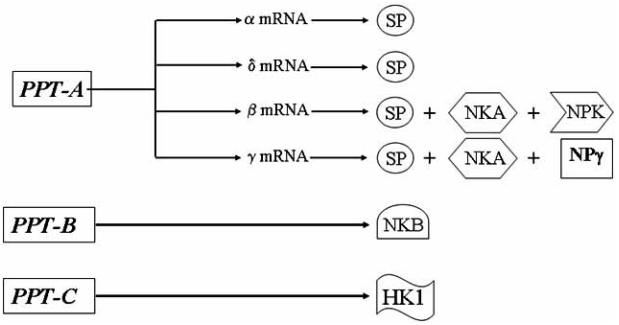

Fig. (2). Cartoon depicting the three mammalian tachykinin genes and their respective spliced variants, and protein products [24, 25].

There are three tachykinin receptors: NK1, NK2 and NK3 [34]. SP, the major PPT-A gene product, binds to each receptor subtype, although with different binding affinity [35]. SP shows binding preference for NK1 while NK-A shows preference for NK2 [34]. Common binding of the tachykinins to the NK receptors appears to be attributed to the common carboxyl-terminus sequence in the tachykinins [33]. NK1 is widely distributed in the brain, and in peripheral tissues, such as cells of the hematopoietic system, mammary epithelium, and endothelium [36]. SP-NK-1 interaction mediates inflammatory responses [37-40]. Although NK1 has been suggested as a drug target for treating psychiatric and neurological disorders, including schizophrenia, anxiety and depression, the current trials are disappointing [41]. The NK1 antagonists have however been approved for the treatment of chemotherapy-induced emesis [42].

NK2 is predominantly expressed in peripheral tissues, and has been found in neural tissues [21]. Studies have been done to identify the relevant residues within NKA that are critical to efficient binding to NK2 [21]. A series of NK2 antagonists that are small polycyclic peptides, including MEN10207 (Asp-Tyr-DTrp-Val-DTrp-DTrp-Arg-NK_2) and MEN10376 (Asp-Tyr-DTrp-Val-DTrp-DTrp-Lys-NK_2) have established Asp^4 and Val^7 as crucial residues for their selectivity as NK2 antagonists [21]. The discovery of NK2 recep-

tor nonpeptide antagonists paralleled that of NK1 with regards to the strategies used in their identification, such as random screening of chemical libraries and targeted drug design through modifications of existing structures [13]. The NK2 receptor antagonist, SR-48, 968 and other classes were identified by random library screen and modification of compounds [43]. In other cases, a different class of stable NK2 antagonists were obtained by incorporating the N-methylamide into six-membered ring lactam 4 ring [44]. Since NK2 receptors are mostly distributed throughout the periphery, as opposed to the central nervous system, in the case of NK1 [34]; Selective nonpeptide antagonists of NK2 raised interest as potential therapies for urinary incontinence, bowel, and airway disorders [45].

NK RECEPTORS AND BREAST CANCER

In humans, NK1 is expressed as two isoforms: full-length and truncated [46]. The truncated NK1 (NK1-Tr) lacks 100 amino acid residues in the C-terminus of the full-length form [47]. Co-expressions of both NK1-FL and NK1-Tr have been detected in breast cancer cells [46]. The full-length NK1 (NK1-FL) appears to be a negative regulator of the NK1-Tr [46]. The truncated NK1 appears to occur at the level of gene transcription rather than translation. This premise is made because both transcripts have been detected in BC [10]. Since the critical region of NK1-Tr that is relevant for receptor internalization is omitted, but the receptor can signal through G-proteins, it has been proposed that signaling without internalization might be partly responsible for the tumor-promoting properties of NK-1Tr [46]. Although the expression of both NK1 isoforms has been confirmed in breast cancer cells, only the truncated receptor has been associated with tumor-promoting properties [46]. Studies performed on primary BC tissue suggest that the NK1-Tr is expressed during an early stage of cancer development [46].

In healthy cells, such as primary bone marrow stroma and non-tumorigenic breast cell lines, *PPT-A* and *NK1* are induced by growth factors whereas *NK2* is constitutively expressed [48]. *NK1* is induced by IL-1α, stem cell factor, GM-CSF, but is inhibited by TGF-β1 [48]. Thus, in healthy cells, the expression of NK1 is inversely proportional to that of NK2 [39]. NK1 has been linked to cell cycle progression and proliferation in bone marrow progenitor cells while NK2 blunts cell proliferation, acting as a negative feedback to NK-1 [48]. Although currently unclear, the biological responses mediated by NK1 and NK2 in healthy cells involve intracellular crosstalk between NK1 and NK2 [48].

BC cells constitutively express *PPT-A*, *NK1* and *NK2* [6]. Their expressions in BC cells resulted in autocrine stimulation, consequently mediating the proliferation of the cancer cells [6]. Enhanced transcription of *NK1* could be due to the activation of the transcription factor NFκB in BC cells, which has been shown to be important for *NK1* induction [49]. Since both NK1 and NK2 mediate the production of growth factors, one can only speculate that these two receptors could be the primary mediator of cancer-producing factors that affect the microenvironment. Factors mediated by NK1 and NK2 include IL-1α, SDF-1α, TGF-β1, and GM-CSF [10, 50]. This would facilitate the survival of the BC cells, and perhaps facilitate their invasion and/or metastasis. These are unresolved mechanisms that require indepth studies. If done, this could lead to multiple drug targets for breast and perhaps other related cancers.

DESENSITIZATION OF NK1 AND NK2

Previous studies have demonstrated that a fast decrease in cell surface binding between SP and NK1 accompanies the rapid internalization of NK1 from the plasma membrane [51]. Moreover, continuous SP binding following receptor internalization, independent of *de novo* protein synthesis, suggests gradual recycling of NK1 to the cell surface [51]. Garland *et al.* demonstrated that while NK1 is promptly desensitized by repetitive exposure to SP, NK2 shows a continuous response to NKA stimulation with diminished receptor desensitization [52]. In a series of experiments, the authors reveal that, under the same experimental conditions, NK1 displays greater desensitization than NK2 when exposed to its respective agonist. Both NK1 and NK2, however, were observed to resensitize within the same time frame [52]. From these studies, it was deduced that internalization occurs through a common mechanism. Receptor internalization requires the formation of early endosomes and the transferin receptor with clathrin molecules acting as mediators [53].

Although the mechanism of NK2 resensitization has not been elucidated, the accepted model of NK1 resensitization requires the release of SP from NK1 in an acidified endosomal compartment prior to their respective sorting into either a degradative or recycling pathway. Moreover, NK1 resensitization requires the action of phosphatases in order to dephosphorylate multiple sites present in the C-terminus and the third intracellular loop of NK1 [52, 54].

Forced internalization of NK1 has been proposed as a mechanism to decrease both local and systemic inflammation [40]. The premise is to prolong desensitization to SP, thereby limiting the production of inflammatory cytokines. However, previous studies have demonstrated that NK1-Tr is resistant to forced desensitization, and cells expressing this receptor isoform display prolonged response to ligand stimulation [10]. Thus, if forced desensitization of NK1 is to be used as a therapeutic tool, the details of the mechanism of NK1-Tr desensitization needs to be clarified by further research.

LIPID RAFTS AND GPCRS

Currently, it is believed that many membrane-bound proteins are structurally organized within lipid rafts [54]. The lipid rafts contain a high concentration of cholesterol, saturated phospholipids, sphingolipids, and glycosphingolipids, which allows for compact packing of integral membrane proteins, such as receptors, within the lipid rafts [54, 55]. The recognition that lipid rafts contain many proteins associated with cellular signaling exposed the fact that these structures are important to the processes of cellular signaling [56].

It is important to note that CXCR4 is localized into lipid membrane rafts on the cell surface. This localization is critical to the function of this receptor [57]. These rafts help to concentrate these receptors along with other molecules important in their function, including CD24 and CD26 [57, 58].

CD26, also known as dipeptidyl endopeptidase, co-localizes with CXCR4 on the cell surface and inactivates SDF-1α before it can bind CXCR4 [59]. The function of CD24 has only recently been discovered. It has been shown to down regulate CXCR4 signal by altering the occupants of lipid membrane rafts such that downstream targets of CXCR4 are not accessible to the receptor [58]. A better understanding of exactly how CD24 affects CXCR4 is important, since $CD44^{+}/CD24^{-/low}$, has been suggested as the phenotype of BC stem cells and it might be important as a drug target along with antagonists to the GPCRs. If CD24 is indeed reduced in BC stem cells, then it should be upregulated in the highly proliferative cells, analogous to cancer progenitors. The highly invasive cancers use CXCR4, and other G-protein coupled receptor, such as NK1 [6, 14]. Co-expression of CD24 and CXCR4 might be regulated differently in malignant cells. A focus on these two membrane proteins need further investigation to study how they could be targeted for BC, in particular the population of cells that are highly proliferative.

In a recent report, Monastyrskaya *et al.* mention that accumulation of NK1, and its signaling partners Gαq, Src, Raf, etc, into lipid rafts, is integral for the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) [58]. According to these authors, the structure of lipid rafts may be implicated in the segregation of the Gαq and Gαs signaling pathways activated by NK1 [58].

Recently, an elegant study on phospholipid phosphatidylinositol 4, 5-biphosphate (PIP_2) signaling, demonstrated strong clustering of NK2 along the plasma membrane. The study suggests that NK2 localizes to lipid rafts [53]. Treatment of cell membranes with methyl-β-cyclodestrin, eliminated the cholesterol molecules associated with lipid rafts, leading to an effective disruption of NKA-mediated phospholipase C (PLC) signaling, through disruption of the constitutive recycling process associated with G-protein coupled receptors [55]. In an independent study, Cezanne *et al.* propose that 30-35% of NK2 receptors are anchored to vesicular precursor regions at the cell surface. These microdomains, are reported to be flat, prior to clathrin polymerization, and are projected to contain about two thousand NK2 molecules [59]. The authors speculate that confinement of NK2 in microdomains might be due to their association with intercellular molecular signal scaffolds referred to as 'transducisomes' [59]. Fig. (**3**) illustrates how potential association of NK1 with transducisomes would allow for rapid, synergistic, activation of second messengers, prior to receptor internalization.

Since the CXCR4, NK1, and NK2 receptors localize to lipid rafts, these rafts may provide novel therapeutic targets for treatment of local and metastatic BC, as reported for the cannabinoid CB1 receptor antagonist for BC [60]. One can envision, for example, targeting the essential clustering of these receptors within lipid rafts. Another potential therapeutic target may be deactivation of an entire lipid raft cluster, containing the receptors in question, through either blocking the receptor/ligand interaction, or through targeting of the intercellular molecular signal scaffolds associated with lipid rafts. The targeting of lipid rafts may potentially have a large impact on secondary signaling cascades, because by targeting the lipid rafts, one can essentially target hundreds, if not thousands of receptors simultaneously.

SUMMARY AND CONCLUSION

Low invasive BC cells have been proposed as those that enter the bone marrow at an early period of the disease. We propose that this could occur prior to clinical detection of the primary tumor. These cells could very well represent the population that resurgences into tertiary metastatic disease

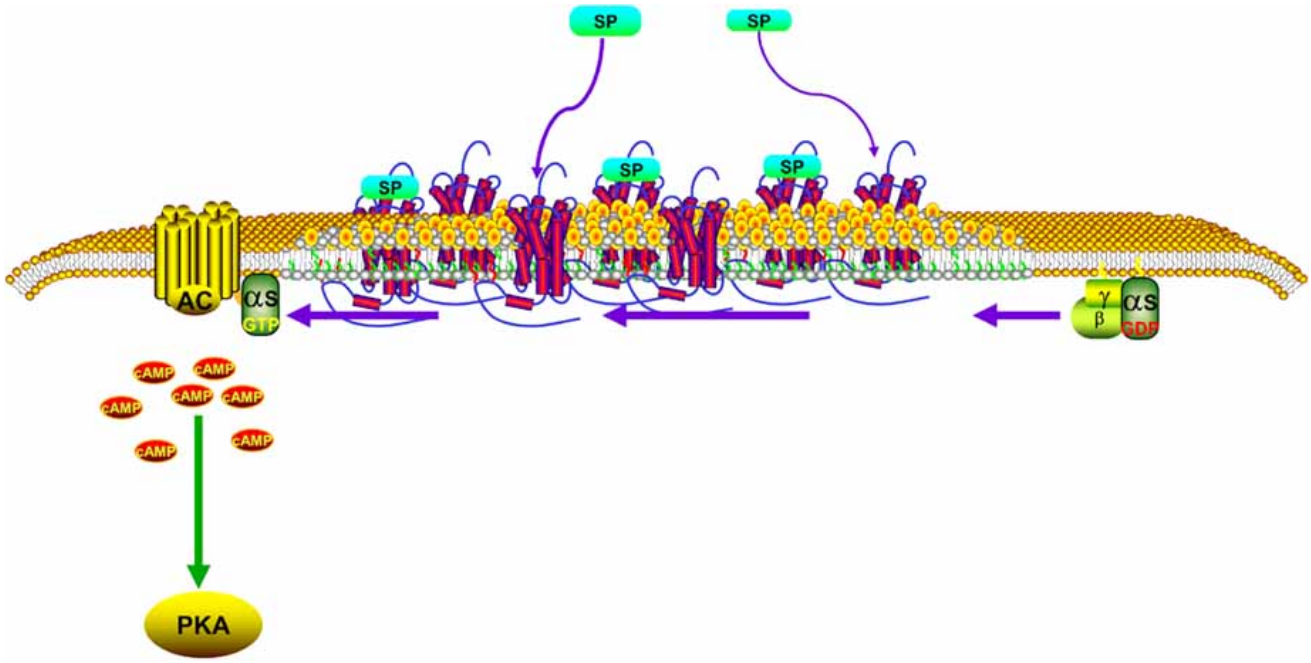

Fig. (3). Clustering of SP receptors, *via* lipid rafts, facilitates a synchronized rapid response to stimulous.

decades after initial remission and/or after they enter the bone marrow. Metastasis remains one of the more pressing issues associated with prevention and treatment of BC, for it is the main indicator of poor prognosis among patients that suffer from this disease [61]. Considering this fact, it is important to understand the mechanisms associated with BC metastasis to the bone marrow, in order to effectively target this aspect of the disease.

It is widely accepted that many downstream effectors of GPCRs are dysregulated in breast cancers. This characteristic, however, may be due to the convergence of multiple GPCR signals on similar pathways, and may not necessarily be the root cause of oncogenic transformation. Therefore, simply targeting a particular GPCR, or one particular pathway, may not inhibit the development of cancer and its growth.

The SDF-1α/CXCR4 signaling pathways have been implicated as the main mechanism for homing of BC cells into bone marrow during late stage metastasis. Our studies have shown that although this pathway is also important in the early metastasis of BC cells to the bone marrow, the key regulator of bone marrow invasion, during this period of time, is the *PPT-A* gene. This renders the neurokinin receptors, particularly NK1 and NK2, as excellent targets for blocking BC entry into the bone marrow. This is accentuated by recent reports that NK1-Tr demonstrates tumorigenic properties in breast tissue. Perhaps, then, it would be useful to complement the standard treatments of BC, with novel approaches aimed at preventing BC invasion of the bone marrow.

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ABBREVIATIONS

SDF-1α = Stromal cell-derived factor 1α

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