Roles of protein kinase C and arrestin in migration of cells via CXCR4/CXCL12 signalling axis

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ABSTRACT:

Aim: The chemokine system not only coordinates leukocyte migration in immunity and inflammation, but it is also implicated in the pathogenesis of many human diseases, including cancer. The expression of chemokines and their receptors is altered in many malignancies and leads to aberrant chemokine receptor signalling. Emerging evidence indicates that the tumour microenvironment has critical roles in all aspects of cancer biology, including growth, angiogenesis, metastasis and progression. One of the important representatives of this system are the chemokine ligand CXCL12 and its receptor, CXCR4 as they are most commonly found on human and murine cancer cells. Our aims are to study and understand if there are any differences in activation of signalling molecules in the downstream signalling cascades in CXC- chemokine receptors in different cell types, and to identify the importance of different effector proteins in migration of cells; the two proteins of interest include Protein Kinase C (PKC) and arrestins.

Methodology: Experimentation was undertaken in MCF-7 breast cancer cells and Jurkat leukemic T-lymphocytes which both naturally express the chemokine receptor CXCR4. Small molecule inhibition and protein overexpression was used in chemotaxis and calcium release assays to measure cellular responses. Immunocytochemistry was used to determine the effect of protein blocking and protein overexpression on receptor internalisation, protein localisation and the formation of cellular structures associated with migration.

Results: Inhibition of PKC has no effect on Jurkat cell migration, but it blocks MCF-7 cell migration showing that there is a difference in the usage of PKC in different cell types. Arrestin 3 is important for migration in both suspension Jurkat cells and adherent breast cancer MCF-7 cells.

Conclusion: Our study shows that CXCL12-induced migration may be arrestin 3 mediated. We have also shown that activation of signalling molecules needed for CXCL12-induced migration can differ between different cell lines. Overall, the research in this thesis has identified potential signalling molecules that can be targeted to interfere with migration of cells.

ABBREVIATIONS:

7TMRs	7 transmembrane receptors
Ab	Antibody
ADP	Adenosine diphosphate
AML	Acute myelogenous leukaemia
ANOVA	Analysis of variance
Arr2 siRNA	Arrestin 2 siRNA
Arr3 siRNA	Arrestin 3 siRNA
ASK1	Apoptosis signalling kinase 1
ATCC	American Type Culture Collection
AT1AR	Angiotensin-II type 1a receptor
ATP	Adenosine triphosphate
BRAK	Breast and kidney-expressed chemokine
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
CCL	C-C motif chemokine receptor ligand
CCR	C-C motif chemokine receptor
CHO.CCR5	Chinese hamster ovary cells, CCR5 stably transfected
CO_2	Carbon dioxide
c-Src	Cellular sarcoma non-receptor tyrosine kinase
Crk	Proto-oncogene c-Crk
CXCL	C-X-C motif chemokine receptor ligand
CXCR	C-X-C motif chemokine receptor
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EC_{50}	Concentration at which 50% if an effect occurs
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase

FAK	Focal adhesion kinase
FCS	Foetal calf serum
Gα	G-Protein α subunit
Gβγ	G-Protein βγ subunit
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G-Protein coupled receptor
Grb2	Growth factor receptor-bound protein 2
GRK	G-protein couple receptor kinase
GTP	Guanosine triphosphate
HEK293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
HSCs	Hematopoietic stem cells
IC_{50}	Concentration at which 50% inhibition occurs
IP3	Inositol triphosphate
IRS-1	Insulin receptor substrate-1
IL	Interleukin
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MAP2K/MEK/MKK	MAPK kinase
MAPKKK/MAP3K	MAPK kinase
MCF-7	Michigan Cancer Foundation-7 (breast cancer cell line)
MKP7	MAPK phosphatase 7
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
p38	p38 mitogen activated protein kinase
PAK	p21-activated kinase
PAR-2	Protease-activated receptor-2
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline

PBMC	Peripheral blood mononuclear cell
pEGFP.C2	Plasmid DNA coding enhanced green fluorescent protein fused to C terminus
pArr2.EGFP	Plasmid DNA coding arrestin 2 fused to EGFP protein
pArr3.EGFP	Plasmid DNA coding arrestin 3 fused to EGFP protein
pA2-Mut.EGFP	Plasmid DNA coding arrestin 2-mutant fused to EGFP protein
pH	Pleckstrin homology
PI	Phosphoinositide
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PTX	Pertussis toxin
Pyk-2	Proline-rich kinase-2
Rac	Ras-related C3 botulinum toxin substrate
RAF	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma GTPase
Rho	Ras homology GTPase
RhoA	Ras homolog gene family, member A
RLuc	*Renilla Luciferase*
RPMI	Roswell Park memorial institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SDF-1α	Stromal cell-derived factor 1α
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
STAT	Signal transducer and activator of transcription
TKs	Tyrosine kinases
TNFα	Tumour necrosis factor alpha
TPA	12-O-tetradecanolphorbol-13-acetate
VEGF	Vascular endothelial growth factor
WT	Wild type

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CHAPTER 1: Introduction

1.1. Introduction

Tumour metastasis is the major cause of death in cancer patients who have suffered from a primary solid tumour. Recent evidence has shown that chemotactic signalling plays an important role in tumour invasion and spreading [1–3]. Studies have shown that selected chemokine receptors are often upregulated in a large number of common human cancers, including those of breast, lung, prostrate, colon and melanoma [4–8]. It is now believed that chemokines and their receptors play key roles in cancer cells migration and metastasis to different organs as they share similarities with leukocyte trafficking to sites of inflammation, including adherence of cells to endothelium, extravasation and migration from blood vessels [3,9]. Infiltrating leukocytes are not the only cells that respond to chemokine gradients in cancers; cancer cells can express chemokine receptors and respond to chemokine gradients [4,10].

Investigation of chemokines and their receptors, and also the downstream signalling pathways that they couple to, may give us a better understanding of how and why particular chemokines contribute to cancer growth and metastasis [11]. Understanding the intracellular signal transduction events for specific receptors will help to identify therapeutic targets and to stop migration of cells, hence stop cancer cells to metastasize. Metastatic tumours generally have poor prognosis, as they are usually difficult to treat with current therapies. The molecular mechanisms underlying metastasis are still poorly understood due to complex network of chemokines and their receptors. The need for novel and specific anti-migratory therapies to treat chronic inflammatory and metastatic diseases is great. Therefore, a greater understanding of signal transduction events triggered by chemokine receptor activation is vital.

CXCR4 is the chemokine receptor that is most commonly found in cancer cells [2,12]. It is found in tumour cells from at least 23 different types of human cancers of epithelial, mesenchymal and haematopoietic origin [1]. CXCR4 expression is generally a characteristic of malignant epithelial cell, as its expression is usually low or absent on normal breast, ovarian and prostate epithelia [1,4,13]. CXCR4 makes an interesting system to study not only due to its expression in numerous inflammatory diseases and

cancers but also its relevance to HIV research as a co-receptor to CC- chemokine receptor, CCR5 [14,15]. CXCL12 is a homeostatic chemokine and is a known ligand for CXCR4 [2]. It is found constitutively expressed in several organs such as lung, liver, skeletal muscle, bone marrow and also at sites of metastasis in breast and thyroid cancer, neuroblastoma and haematological malignancies [4,16,17].

The binding of CXCL12 to CXCR4 initiates divergent signalling pathways downstream of ligand binding. This usually results in a variety of responses, including chemotaxis, increase in intracellular calcium and gene transcription [11,18]. Although the chemokine system has been heavily studied over the last two decades and is considered to be highly promising drug targets for immunological and inflammatory diseases, current studies have not yet resulted in many new therapeutics. This is likely due to a complexity of the chemokine system, that is characterised by redundancy, pleiotropy and differences among species [19].

1.2. G-Protein Coupled Receptors (GPCRs)

G-protein coupled receptors (GPCRs,) also known as 7 Transmembrane Receptors (7TMRs), represent the largest family of membrane receptors. GPCRs mediate most of our cellular responses to hormones, neurotransmitters and environmental factors, and so they have been comprehensively studied over these past three decades due to their great potential as therapeutic targets for a broad spectrum of diseases [20]. GPCRs in vertebrates are commonly divided into 5 families based on their sequence and structural similarity [21]: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste2. The rhodopsin family is the largest and most diverse family, and the members are characterised by conserved sequence motifs [20]. It is apparent that they share similarities in structures, with the exception of functionally unique conserved motifs, but the level of sequence homology is relatively low [22], resulting in individual GPCRs having unique combinations of signal-transduction activities involving multiple G-protein subtypes, and G-protein-independent signalling pathways and complex regulatory processes [20].

G-proteins, also known as guanine nucleotide-binding proteins, are coupled to the receptor through its C-terminus segment and the third intracellular loop [23]. G-

proteins are important signal transducing molecules in cells, known to function as molecular switches inside cells, and there are two classes of G-proteins; the first class function as monomeric small GTPases, while the second class function as heterotrimeric G-protein complexes [24]. Heterotrimeric G-proteins are the molecular switches that turn on intracellular cascades in response to the activation of the GPCRs by extracellular stimuli, hence, they have a crucial role in defining the specificity and temporal characteristics of the cellular response [25]. All GPCRs couple to heterotrimeric G-proteins (Gα, Gβ/Gγ subunits), which become activated upon ligand binding and transduce signals via a wide range of intracellular molecules. Studying the ability of GPCRs to activate selective signalling pathways according to the conformation stabilised by bound ligands and the duration of GPCR signalling is often important and a more complex concept in the GPCR field [26].

GPCRs identify many extracellular signals and transduce them to heterotrimeric G-proteins, which further transduce these signals intracellularly to appropriate downstream effectors. These receptors play an important role in various signalling pathways, including chemotactic migration, by processing complex but subtle physiological environments and transduce signalling into intracellular responses such as cell survival and/or proliferation, chemotaxis, intracellular calcium release and gene transcription [2,27].

1.3. Chemokines and chemokine receptors

Chemokines are a family of chemotactic cytokines and can be induced by inflammatory cytokines, growth factors and pathogenic stimuli [28–30]. Chemokine signalling is involved in gene transcription, in cell invasion, motility, survival and can coordinate cell movement during inflammation and homeostatic transport of haematopoietic stem cells (HSCs) and leukocytes such as lymphocytes and dendritic cells [1,31]. Directed cell migration of cells that express the appropriate chemokine receptor occurs along a chemokine gradient, causing them to move towards a high concentration of chemokines [32–34]. Chemokines compromise a superfamily of about 50 human ligands and there are at least 20 seven-transmembrane-domain chemokine receptors, belonging to the family Class A G-protein coupled receptors (GPCRs) [29,35].

Chemokines and their receptors have been divided into four families on the basis of the pattern of first two cysteine residues that are within the amino terminus in the ligands; CXC-, CC, C- and CX3C- [1]. They have been classified as being 'homeostatic' – controls leukocyte navigation during immune surveillance, and 'inflammatory' – controls cell recruitment to sites of infection and inflammation [36]. Chemokines are key regulators of leukocyte migration that ensure localisation of cells to tissues, depending on their activation state and inflammatory state of the tissue [37]. The profile of chemokine-receptor expression on an individual cell is determined by its family, stage of differentiation, and microenvironmental factors such as chemokine concentration, presence of inflammatory cytokines and hypoxia [1].

Most chemokines bind to multiple receptors and the same receptor may bind to more than one chemokine; for example, CXCL12 binds to CXCR4 and ACKR3 (also known as CXCR7), and CCL3 binds to CCR1, CCR2 and CCR5 [1,2]. Differences in cytoplasmic signalling are governed by factors such as the number of phosphorylation sites the receptor expresses [15]; for example, CCR5 possesses 7 sites while CXCR4 possesses 21. Comparisons and distinctions between the structures and functions of different chemokines and GPCR families are important for identifying cell-, receptor- or family-specific signalling. This information can then be used to identify therapeutic targets for inflammatory diseases and cancers where specific chemokine receptors are known to be involved. Different chemokines can elicit other important cellular responses [38,39] and/or activate different pathways to elicit a particular response [40,41].

Chemokines have an important role in the development and maintenance of innate and adaptive immunity [12]. Chemokines and their receptors play a key role in development and homeostasis as well as in several inflammatory diseases and cancers. Chemokines also have a role in embryonic development [42,43]; current research involves chemokine receptor CXCR4 in the maternal decidualized stromal cells [44].

Chemokine receptors are critical regulators of cell migration in terms of immune surveillance, inflammation and development [45].They share a seven transmembrane-spanning α-helix architecture, separated by alternating intracellular and extracellular loop regions, that accommodates signal propagation from across biological membranes [20,46].

Most tumours express chemokine receptor CXCR4 at higher levels than normal corresponding tissue (table 1); other receptors include CCR6 and CX3CR1 which are overexpressed in colorectal and pancreatic cancer [47,48], CXCR2 and CCR10 in melanoma [49,50] and CXCR6 in prostate cancer [51].

Table 1.1: Expression of CXC chemokine receptor 4 (CXCR4) in cancer metastasis

Cancer cell type	Site of metastasis	References
Breast	Lung, Lymph Node	[4,52]
Prostate	Bone	[53]
Non-small cell lung	Pleural Space	[54]
Ovarian	Peritoneum	[13,55]
Pancreas	Liver, Lung	[56]
Melanoma	Lymph Node	[57]
Neuroblastoma	Bone, Bone Marrow	[58]
Oesophageal	Lymph Node, Bone Marrow	[59]
Colorectal	Liver	[60]
Osteosarcoma	Lung	[61]
Renal	Adrenal Glands, Bone	[62]
Gastric	Peritoneum	[63]

The expression of CXCR4 on malignant epithelial cells implies that the CXCR4/CXCL12 pathway plays an important role in directing the metastasis of $CXCR4^+$ tumour cells to organs that express CXCL12, which include lymph nodes, lungs, liver and bones [2]. Several $CXCR4^+$ cancers metastasize to the bones and lymph nodes in a CXCL12-dependent manner, in which, the bone marrow can provide a protective environment for tumour cells [9,64].

Due to high levels of expression of CXCR4 in cancer cells coupled with the fact that CXCR4 in cancer metastasis leads to a poor prognosis, the CXCR4/CXCL12 signalling axis serves as an attractive target for cancer metastasis therapy, hence, using and understanding CXCR4 as a model system is important and interesting.

1.4. Activation of chemokine receptors

Upon activation by a chemokine ligand, the GPCR chemokine receptor undergoes a conformational change and activates the G-proteins. Heterotrimeric G-proteins are composed of three subunits; α, β and γ, and their switching function depends on the ability of the G-protein α subunit (Gα) to cycle between an inactive GDP-bound conformation that is primed for interaction with an activated receptor, and an active GTP-bound conformation that can modulate the activity of downstream effector proteins [25]. Conformational changes in the cytoplasmic domains of the receptor and activation of G-proteins leads to the dissociation of G-protein subunits β/γ (Gβγ) dimer from Gα [65]. Both these subunits complexes then act upon their downstream effectors and thereby initiate intracellular signalling processes, such as changes in the cytoskeleton and transcription of genes involved in cell migration and survival [66]. Protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), Rho and mitogen-activated protein kinase (MAPK) are amongst the examples of proteins that may be activated by chemokine receptors [66,67]. One of the major functions of chemokines is lymphocyte trafficking, and one of the pathways that mediates this process involves PI3K activation. PI3K can be activated by both Gα and Gβγ subunits. PI3K activation can result in the phosphorylation of several focal adhesion components such as proline-rich kinase-2 (Pyk-2), focal adhesion kinase (FAK), paxilin and adaptor protein, proto-oncogene c-Crk (Crk), which has been found to play a role in mediating cell migration [2].

Receptor desensitisation and internalisation by agonist-dependent phosphorylation of the C-terminal tail of the GPCR by G-protein receptor kinases (GRKs) then occurs to refract from continued stimuli [68]. Receptor phosphorylation subsequently promotes binding of arrestin, which sterically block further interaction with G-proteins and mediate receptor internalisation through clathrin coated pits [69]. Endocytosis of a GPCR can lead to either lysosomal degradation or recycling back to the cell surface and resensitisation [11]. After signal propagation, the GTP of Gα-GTP is hydrolysed to GDP and Gα becomes inactive (Gα-GDP), which leads to its re-association with the Gβγ dimer to form the inactive heterotrimeric complex [27].

There are many classes of Gα subunits and some examples include $G_S\alpha$ (G stimulatory), $G_i\alpha$ (G inhibitory), $G_O\alpha$ (G other), $G_q\alpha$ and $G_{12/13}\alpha$ and they behave differently in the

recognition of the effector molecule but share a similar mechanisms of activation [70,71]. As discussed in section 1.2, GPCRs have been best characterised with respect to signalling through heterotrimeric G-proteins, primarily involving $G_i\alpha$ subunit (figure 1.1) [11]. With the use of *pertussis* toxin (PTX), it has been shown that most CXCR4 activation is dependent on activation of $G_i\alpha$ proteins as PTX, which ADP-ribosylates $G_i\alpha$, specifically inhibits GPCR/$G_i\alpha$ coupling [66]. $G_i\alpha$ proteins mainly inhibit adenylate cyclase and transduce signals through tyrosine kinases such as Src [72].

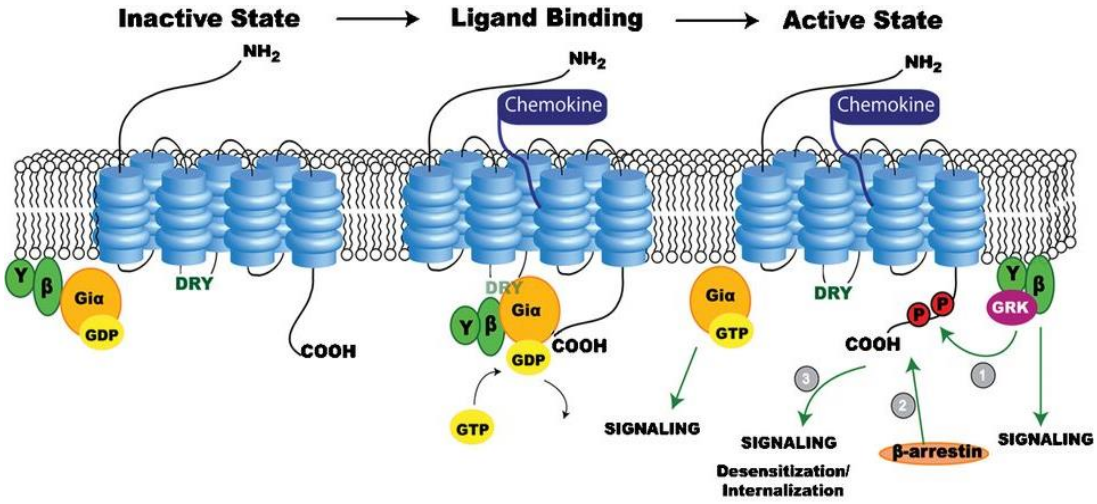


Figure 1.1 (Adapted from O'Hayre et al. (2008) [11]): Simplified overview of GPCR signalling which represents the most upstream events after receptor activation common to all GPCRs. Receptor activation and signalling via heterotrimeric G-proteins, which were activated by exposing important motifs such as the DRY motif (TM-III) [73], followed by desensitisation through GRKs and arrestins. GPCRs may have complex regulatory processes by numerous unique combinations of signal-transduction involving multiple G-protein subtypes, and G-protein-independent signalling pathways via downstream effectors such as GRKs and arrestins.

Chemokine activity is often determined by measuring the transient increase in cytosolic free calcium [74]. Ligand stimulation results in an increase in intracellular calcium, which is not only achieved via $G_q\alpha$, but also via the dimeric complex, $G\beta\gamma$. $G\beta\gamma$ subunits are crucial for the activation of many chemokine-induced pathways. $G\beta\gamma$ triggers PLC activation and formation of inositol triphosphate (IP3) and diacylglycerol (DAG), resulting in mobilisation of calcium from intracellular stores ultimately leading to the regulation of processes such as gene transcription, cell migration and cell adhesion [70]. The other major $G\beta\gamma$ activated pathway is PI3Kγ [72]. There is growing

evidence that Gβγ are responsible for signalling to chemotaxis downstream of various chemokine receptors [75–78].

The signalling and physiological response downstream of receptor activation can vary, depending on chemokine/receptor combination, the cell type and pathophysiological state [38,39]. Chemokine receptors have also recently been shown to be able to transduce signal through G-protein-independent pathway, for example, the arrestin-dependent pathway, through the formation of signalling scaffolds that can lead to specific and independent cellular responses [79–81], hence the precise downstream events leading to signalling are still poorly understood despite extensive study of chemokines and chemokine receptors.

As discussed above, malignant cells from different cancer types have different profiles of chemokine-receptor expression, but CXCR4 is most commonly found. Upon activation of chemokine receptor CXCR4, a number of signalling pathways are activated leading to a variety of biological responses [82] and they can happen via both G-protein signalling [66] and G-protein-independent signalling involving other proteins such as arrestins and Janus kinases (JAK) [83,84]. Other than emerging evidences showing CXCR4 is involved in inflammation and cancer [12], the complexity in regulation of CXCR4 signalling makes CXCR4 an interesting system to study and understand.

1.5. CXCR4/CXCL12 in cancer metastasis

The involvement of chemokines and their receptors in cancer, particularly metastasis, has been firmly established [1,4,85] and this is not surprising as tumour metastasis shares many similar features with normal cell migration and leukocyte trafficking [10,85,86]. Chemokines and their receptors provide directional cues for leukocytes or tumour cells for migration and metastasis [87]. The role of chemokines and their receptors in cancer may involve providing directional cues for migration, shaping the tumour microenvironment and/or providing growth signals (figure 1.2) [88–90].

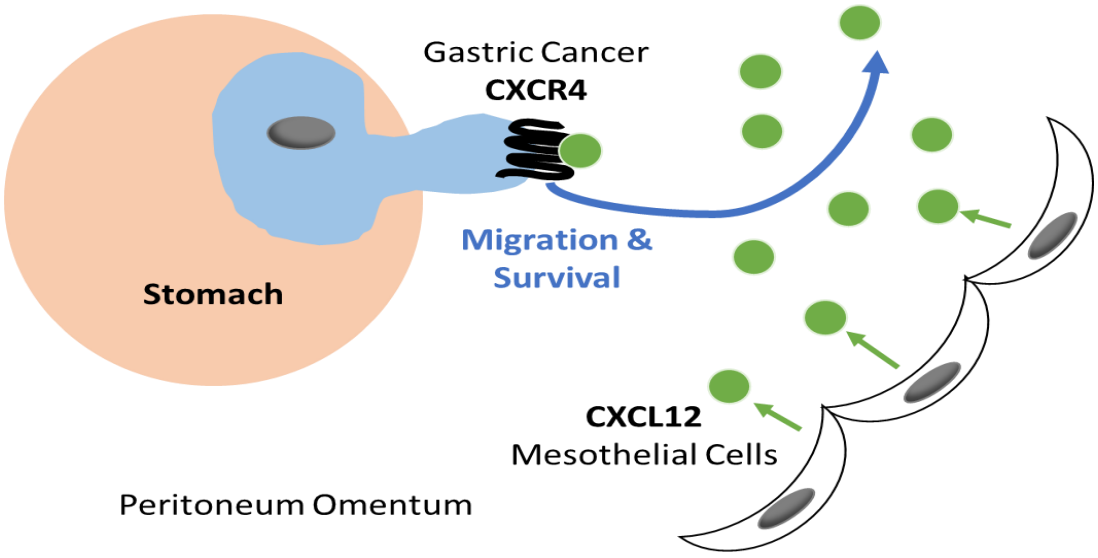


Figure 1.2 (Adapted from Koizumi et al. (2007) [9]): A simplified example of molecular mechanisms of CXCR4/CXCL12 signalling axis that promotes the development of peritoneal carcinomatosis. Gastric tumour cells that express CXCR4 are preferentially attracted to the peritoneum cavity where CXCL12 is produced abundantly. CXCL12 produced in a paracrine manner may also shape tumour microenvironment for the gastric tumour cells to grow and survive [9,91,92].

CXCR4 selectively binds to CXC chemokine, CXCL12, which is also known as Stromal Cell Derived Factor 1 (SDF-1) [21,30]. CXCR4 and CXCL12 play an important homeostatic function by mediating the homing of progenitor cells to the bone marrow and regulating their mobilisation into peripheral tissues upon injury or stress [93]. CXCR4 has been intensively studied in different autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis, and cancers as it is overexpressed in more than 23 cancers and it is involved in leukocyte chemotaxis in inflammatory conditions [3,94,95]. In malignant cancerous tumours, signalling by CXCR4 might be transiently increased by factors such as hypoxia [96], vascular endothelial growth factor (VEGF) [97] and oestrogen [98].

Mukarami *et al.* (2002) and Cardones *et al.* (2003) have demonstrated that overexpression of CXCR4 in luciferase-expressing B16 murine melanoma cell results in enhanced lung metastasis following tail-vein injection in wild-type mice [99,100]. Under stress conditions, endothelial CXCL12 may trigger the arrest of circulating cells by activating adhesion molecules in a manner analogous to the process described in leukocytes [50]. Several groups have shown that CXCL12 stimulates cancer cell proliferation [100,101], migration and invasion [102,103].

Following binding of CXCL12, CXCR4 activates several key migratory pathways, proliferative, and survival signalling pathways, including MAPK pathway, PI3K-Akt pathway, and JAK/STAT pathway [104–106]. There is emerging evidence that in addition to their role in signal termination, arrestins are able to function as signal transducers by activating pathways such as Akt, PI3K and MAPK and also, to act as scaffolds for a number of signalling molecules, resulting in chemotaxis [107–109].

Although there is numerous research on chemokines and chemokine receptors in cancer and tumour metastasis, the exact mechanisms of action are not well understood, and the underlying complexity of the chemokine network makes it difficult to characterise them. Considering some of these complexities, it may be crucial to elucidate more precise mechanisms, and thus enable the development of better cancer therapeutics to stop migration of cells.

1.6. Downstream signalling transduction via CXCR4/CXCL12 signalling axis

Upon CXCL12 binding to the CXCR4 receptor, signalling is transduced by activation of the heterotrimeric G-proteins, which is composed of Gα, Gβ and Gγ subunits. As discussed previously in section 1.4, both Gα and Gβγ complexes subsequently induce chemokine signalling and regulate a wide variety of downstream pathways that ultimately lead to the physiological responses, such as chemotaxis [110]. Chemokine receptor signalling in migration and survival/proliferation occurs through the class IB PI3Kγ, which activates Rac and subsequently PAK (p21-activated kinase). Activation of FAK, pyk2 and other tyrosine kinases are also important in this process [111]. Anti-apoptotic signalling, transcription of growth and proliferation-related genes, and transcription of matrix metalloproteinases (MMPs) involved in migration and remodelling the microenvironment are all transduced downstream from Akt, extracellular signal-regulated kinase (ERK), PKC and tyrosine kinase, such as Src, activation [70,112]. CXCR4 signalling has also been shown to involve the Ras-activated signalling pathway, several src-related kinases such as Src, Lyn, Fyn and Lck, T-cell activating molecule ZAP-70, and vav and small GTPases [2].

Signal transduction is terminated by phosphorylation of the receptor at its cytoplasmic tail by GRKs and subsequent receptor desensitisation and internalisation. The GTP is

rapidly hydrolysed to GDP resulting in re-association of the receptor and the trimeric G-protein complex; the inactive form of the α-subunit (Gα-GDP) is regenerated, thus allowing reassociation with a Gβγ dimer to form the "resting" G-protein, which can again bind to a GPCR and await activation.

As discussed previously , there are different classes of Gα subunits; $G_S\alpha$, $G_i\alpha$, $G_O\alpha$, $G_q\alpha$ and $G_{12/13}\alpha$. $G_i\alpha$ G-proteins inhibit adenyl cyclase as well as activate the Src family of tyrosine kinases while $G_S\alpha$ subunit stimulates adenyl cyclase [2,66]. $G_i\alpha$ has also been shown to stimulate MAPK activation whereas $G_{12}\alpha$ is associated with low molecular weight G-proteins such as Rho and Ras [71,113].

$G_q\alpha$ family acts via PLC, and activate phosphatidylinositol-specific phospholipases, which hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, IP3 and DAG. IP3 and DAG increase the intracellular concentrations of free calcium and activate a number of protein kinases, including PKC and MAPK [70,114,115].

Researchers have shown that CXCR4 may not just be limited to $G_i\alpha$, but CXCR4 can also couple to $G_q\alpha$, $G_O\alpha$ and $G_S\alpha$ [116,117]. Following CXCL12 binding, CXCR4 promotes the recruitment of GRKs that induce site-specific phosphorylation at the C-terminus, which results in binding of arrestins to the phosphorylated receptor [66]. Arrestin recruitment leads to the uncoupling of CXCR4 from G-protein and it induces receptor internalisation [84]. Other than GRKs, PKC has also been observed to directly phosphorylate the CXCR C-tail in different serine residues leading to receptor desensitisation and internalisation [66,68,118]. In addition to their classical role as signal terminators, the recruitment of arrestins to CXCR4 also serves as a scaffold for a number of downstream effectors, MAPK and ERK, and can function as a signal transducer itself by activating pathways such as Akt, PI3K and NF-κB, which can lead to numerous cellular responses such as cell migration [81,109].

CXCL12-induced migration is mediated by PI3K and as discussed previously, PI3K can be activated by Gβγ and also Gα subunits [70]. PI3K activation can result in phosphorylation of several focal adhesion components such as pyk-2, Crk-associated substrate (p130Cas), FAK, paxillin, Nck, Crk and Crk-L, activating intracellular

signalling cascades [111,119]. PI3K can also lead to the activation of serine-threonine kinase Akt, which has been found to play a key role in tumour cell survival [120]. Other than Akt, both p38 and ERK1/2 have also been implicated in tumour cell survival and CXCL12 can promote cell survival through PI3K and MAPK cascades without cell cycle progression [121]. Genes associated with cell survival can be up-regulated upon CXCL12 exposure via post-translational inactivation of the cell death machinery and an increased transcription of cell survival-related genes [122].

Activation of the JAK/STAT pathway by CXCR4 has been proposed to be G-protein-independent, where CXCL12 induces the transient association of JAK2 and JAK3 with CXCR4 and leads to the activation and nuclear translocation of a number of STAT proteins [83,123].

GPCR oligomerization has been hypothesised to play a role in modulating GPCR signalling, and both CXCR4 homodimers and heterodimers have been reported [124]. The functional consequences of homo- or heterodimerization are currently poorly understood. However, homodimerization of CXCR4 has been suggested to be necessary to result in JAK/STAT signalling pathway as well as in enhancement of response to CXCR4 to CXCL12 [66,116]. Even though there are still gaps in knowledge of oligomerization of CXCR4, it should be considered that oligomerization may be a way of regulating signalling while also allowing for alternative signalling pathways upon activation [125].

Given the complex role CXCR4 plays in diverse processes from development to cancer metastasis, CXCR4 is a very intriguing therapeutic target. Due to the intricacy of the roles and network of chemokines and its chemokine receptors, a detailed basic understanding of chemokine-receptor activation is still lacking, even though ample body of work has been done in delineating potential pathways that mediate specific effect, including chemotaxis. Understanding the downstream signalling cascades of CXCR4 upon CXCL12 activation leading to chemotaxis should provide insight into potential therapeutic targets in this pathway.

1.6.1. Downstream effector: Protein Kinase C (PKC)

Protein kinase C (PKC) is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins, which is a fundamental component of cell signalling resulting in crucial roles in most signal transduction cascades [126,127]. PKC activation is dependent on the generation of second messengers DAG and calcium ions, and play important roles in several signal transduction cascades including cell growth and proliferation and also immunological regulation [128–130].

The PKC family consists of 15 isoforms in humans and the isoforms are divided into three subfamilies, based on their second messenger requirements [131]. The three subfamilies consist of (i) conventional (or classical – α, βI, βII, γ), which require calcium ions, DAG and phosphatidylserine for activation, (ii) novel (δ, ε, η, θ), which require only DAG and not calcium ions for activation and (iii) atypical (ζ, ι, λ), which require neither calcium ions nor DAG for activation [130].

All PKCs consists of a regulatory domain and a catalytic domain hitched together by a hinge region. The catalytic region is highly conserved among the different isoforms, and the regulatory region results in the second messenger requirement differences between the isoforms, which are usually similar within the classes [132].

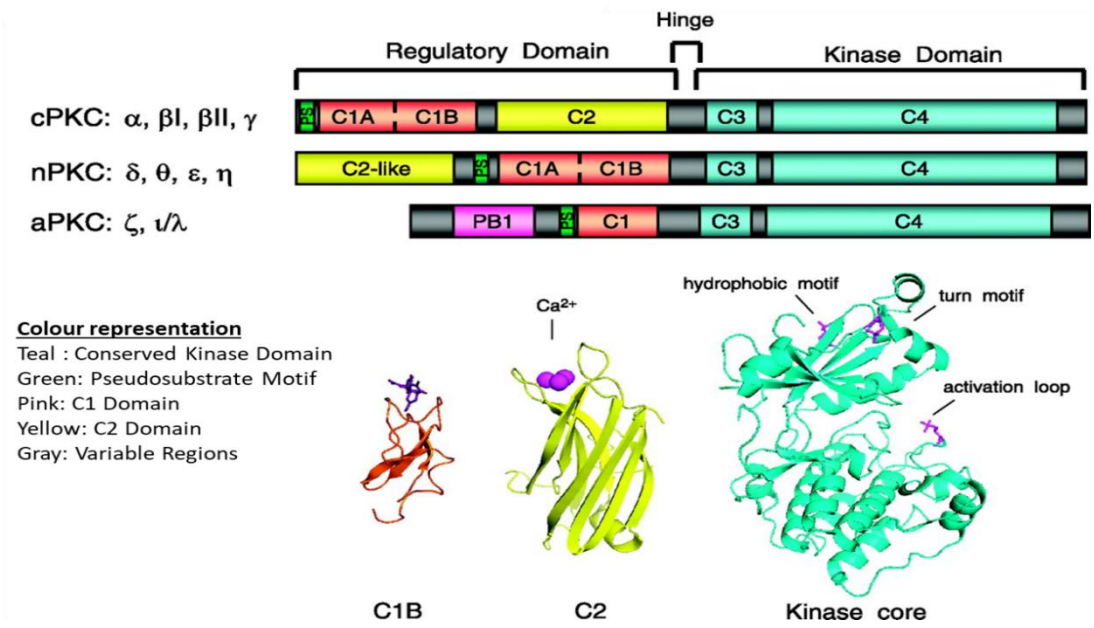


Figure 1.3 (Adapted from Steinberg et al. (2008) [132]**): Domain structure of PKC isoforms** - showing autoinhibitory pseudosubstrate (PS) segment, C1 domains, C2 domains and kinase core; the activation loop, turn motif, and hydrophobic motif phosphorylation sites are indicated. Crystal structures of C1B, C2 and kinase domains are also shown above.

Figure 1.3 shows the domain structure of different PKC isoforms. PKCs have a conserved kinase domain and more variable regulatory domains. All PKC regulatory domains have a pseudosubstrate motif amino terminal to the C1 terminal [132].

As a tool for activating PKC, phorbol esters, derived from oil of the seed of the plant *Croton tiglium*, are much more potent than DAG: they bind PKC with a higher affinity than DAG, and are not readily metabolised [133]. In conventional and novel PKCs, tandem C1 domains are the molecular sensors of phorbol 12-myristate 13-acetate (PMA)/DAG whereas in single atypical PKC, C1 domain does not bind DAG and PMA [134]. The C2 domains function as calcium-dependent phospholipid binding modules in conventional PKCs but not in novel PKCs as novel PKCs do not bind calcium. This difference in C2 domain structure reflects the distinct pharmacology in conventional and novel PKC isoforms [132]. PKCs are regulated by two distinct mechanisms: by phosphorylation, which regulates the active site as well as the subcellular localisation of the enzyme and by second messengers, which promote PKC's membrane association, resulting in pseudosubstrate exposure [135]. Understanding the structural determinants that dictate PKC isoform-specific differences is important in studying

protein-protein interactions, cellular signal transduction and downregulation of receptors [132,136].

PKC are central components in intracellular networks that regulate a vast number of cellular processes including migration [137], and one example is that PKCε regulates neuroblastoma cell motility. Downregulation of PKCε by siRNA suppresses both basal and 12-O-tetradecanoylphorbol-13-acetate (TPA) induced migration [138]. Another study linked PKC to regulation of the actin cytoskeleton, thereby affecting cellular migration and indicating that integrins are crucial mediators both upstream and downstream of PKC in inducing morphological changes [139].

The choice of ligand for receptor activation depends on the cell type and its receptor/signalling pathway signature. Hence, studying PKC activation in chemokine/receptor signalling cascades may be important in understanding the downstream signalling cascades which would lead to cell growth and/or proliferation, gene transcription and regulation of immunological responses [126,136]. Studying PKC signalling and the role of PKC in chemokine/receptor systems can be done by using small molecule inhibitors to see how phosphorylation by these enzymes would affect different processes; examples of inhibitors that were used are included in table 1.2.

Agonist binding to a chemokine receptor activates catalytic cascades of intracellular mediators which greatly amplify the response to an extracellular stimulus. This then causes functional uncoupling of the heterotrimeric G-protein from the receptor and rapid homologous desensitisation consequently occurs [140]. On a molecular level, phosphorylation of ligand-occupied receptors by GRKs or by second messenger activated kinases such as PKC, have been shown to attenuate receptor interaction with G-proteins [141,142]. Recently, it has been revealed that there is increased phosphorylation of Ser339 of CXCR4 following CXCL12 stimulation [143]. Increased phosphorylation of Ser339 was also observed following epidermal growth factor (EGF) or phorbol ester treatment, suggesting that this may be a potential PKC phosphorylation site. GRK2 [14,144] and GRK6 [145,146] have been implicated in the involvement of phosphorylation of CXCR4 upon CXCL12 activation. However, GRK2 has also been suggested to negatively regulate CXCR4 downstream signal transduction,

possibly via MAPK [147]. These data imply that there may be multiple kinases regulating CXCR4 in response to CXCL12 stimulation.

Table 1.2: Small-molecule active site inhibitors

Compound	Type of Inhibitor	Determination of binding	IC50	References
GF109203X	Conventional, Novel PKC inhibitor	Non-selective bisindolylmaleimide inhibitor which has been used effectively in platelets, Swiss 3T3 fibroblasts and macrophages.	α – 8.4 nM βI – 18 nM δ – 210 nM ε – 132 nM ζ – 2.8 μM (10 μM ATP)	[148,149]
Staurosporine	Conventional, Novel PKC inhibitor	Non-selective indolocarbazoles inhibitor which is also a model apoptosis inducer.	cPKC & PKCε – 11 – 32 nM	[150]
Rottlerin	Conventional, Novel PKC inhibitor	Non-selective mitochondrial uncoupler that depolarises the mitochondrial membrane potential, reduces cellular ATP levels. Although there was extensive published documentation to support the use of rottlerin as a selective PKCδ inhibitor, there has been more controversy in the literature over this claim.	α, β, γ – 30 – 42 μM δ – 3 – 6 μM ε, η, ζ – 80 – 100 μM	[151,152]
CID755673	PKD inhibitor	Blocks PKD-mediated protein transport.	PKD1 – 180 nM PKD2 – 280 nM PKD3 – 227 nM PKC - >10 μM	[153,154]

1.6.2. Downstream effector: Arrestins

Arrestins are a small family of proteins important for regulating signal transduction from GPCRs [109,155]. In response to ligand stimulation, GPCRs activate heterotrimeric G-proteins as discussed above. GRK phosphorylation would then occur to prepare the activated receptor for arrestin binding [156,157]. Arrestin binding to the receptor blocks further G-protein-mediated signalling and targets receptors for internalisation [158]. Other than the classical role as signal terminators, arrestins can also act as signalling scaffolds for cytoskeletal signalling molecules and redirect signalling to alternative G-protein-independent pathways [152,153]. Plasma membrane translocation of arrestins to activated receptors and its signalling capabilities even without G-protein-receptor complex strongly suggest the existence of specific receptor conformation for arrestin binding [161,162].

Arrestins are highly flexible proteins that can assume several distinct conformations, and in receptor-bound conformation, arrestins have a higher affinity for a subset of partners which explains how receptor activation regulates arrestin-dependent signalling via arrestin recruitment to GPCRs [163]. Arrestin family contains four members; the two visual subtypes found in retinal photoreceptors are arrestin 1 and arrestin 4 and the two non-visual subtypes are arrestin 2 and arrestin 3 (also known as β-arrestin-1 and -2), which are ubiquitously expressed in all cells [164].

Studying the regulation of intracellular signalling pathways is important as this mechanism regulates aspects of cell motility, chemotaxis and apoptosis but it is still poorly understood. The mechanism may involve recruitment, activation and scaffolding of cytoplasmic signalling complexes via two multifunctional adaptor and transducer molecules, arrestins 2 and 3 [109]. According to numerous studies, there are two hypotheses regarding the requirement for arrestins in chemotaxis; firstly, desensitisation and recycling of chemotactic receptors which are essential for maintaining polarity and secondly, arrestins serve as signalling scaffolds to localise molecules involved in cytoskeletal reorganisation [165–167].

Growth factors, neuropeptides, peptides, hormone and proteases promote cell migration through the activation of their cognate GPCRs or receptor tyrosine kinases

[168]. Chemotaxis mediated by 2 different $G_q\alpha$-coupled receptors, the protease-activated receptor-2 (PAR-2) and the angiotensin-II type Ia receptor (AT1AR), were shown to be arrestin-dependent [169,170]. In both examples, arrestin-dependent chemotaxis does occur independent of G-protein coupling.

According to several studies, arrestins may also trigger signal transduction, including the activation of Src family tyrosine kinases [108], PI3K/Akt [171], insulin receptor substrate-1 (IRS-1) [172], RhoA [173,174] and NFκB [175–178]. In recent years, there is emerging evidence that arrestins serve as scaffolds for numerous signalling networks, including the MAPK pathway [179]. The new paradigm implies that arrestins not only mediate desensitisation of G-protein signalling, but also act as a signal transducer. An example of an arrestin-dependent signalling system is activation of MAPK, ERK [169,170]. Studies indicate that arrestins can sequester active ERK1/2 at the leading edge during PAR2-induced cell migration [180]. It is implied that this facilitates specific phosphorylation of proteins involved in chemotaxis, but such targets have not been identified.

Similarly, it has been demonstrated that arrestin 3 scaffolds c-Jun N-terminal kinases 1/2 (JNK1/2) with its upstream kinases MKK4 and MKK7, which phosphorylate different residues in its activation loop [181]. Activation of p38 signalling cascades is also arrestin-dependent, although a direct scaffolding complex of arrestin and p38 has not yet been established [182–184]. CXCL12-induced chemotactic response, which is arrestin-dependent, is sensitive to p38 inhibitors [182] but these data only show some cellular functions regulated by arrestins involving p38, even though direct arrestin-dependent p38 phosphorylation is not documented. There are a few hypotheses as how arrestins act as scaffold proteins: they form a discrete signalling module, they localise the grouped components to specific areas in the cell and they insulate the active kinases from de-phosphorylation by phosphatases [109]. Arrestin-mediated activation of ERK appears to be linked to the function of arrestins in mediating endocytosis of receptors in clathrin-coated pits, for example, arrestin scaffolded signalling complex has been implicated in internalisation with class B receptors such as AT1AR and ultimately is found in endocytic vesicles together with the receptor [185].

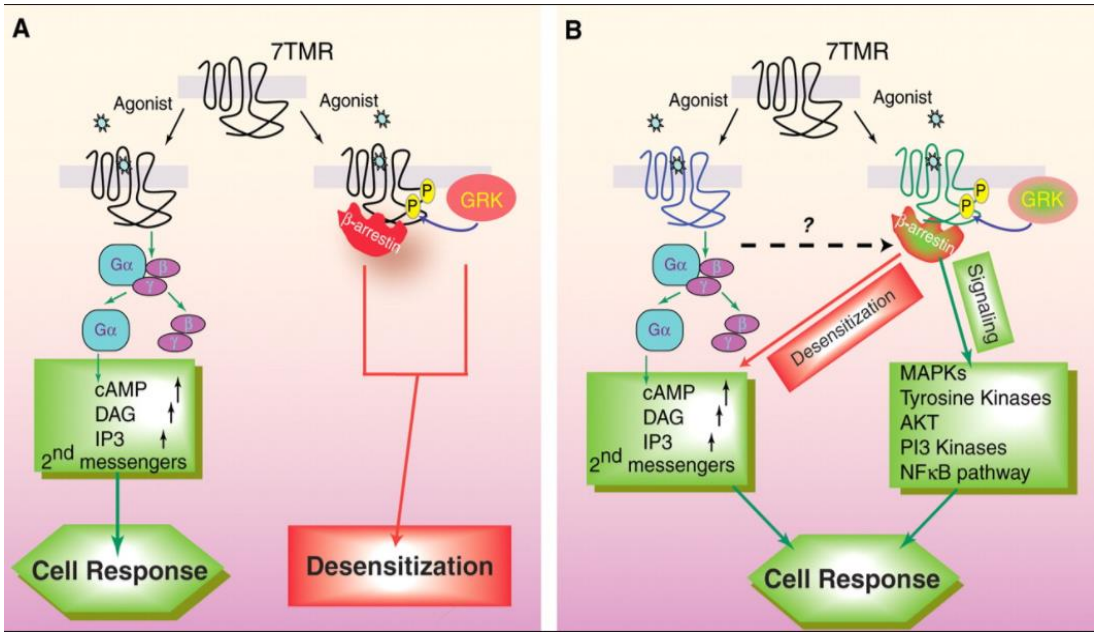


Figure 1.4 (Adapted from Lefkowitz et al. (2005) [109]): Signal transduction by GPCR. (A) Classical pathway where the active form of the receptor stimulates G-proteins and is rapidly phosphorylated by GRKs, which leads to arrestin recruitment and termination of signal by desensitisation. (B) New theory on the role of arrestins which not only mediate desensitisation of G-protein signalling but also act as signal transducers. Another theory of arrestins' new role is arrestin as a scaffolding protein (not shown in diagram).

The requirement of arrestins in chemotaxis downstream of multiple receptors suggests that arrestins play a key role in apoptosis [186–189], inflammation [81,179,190] and mediating cell migration [169,174,191,192]. Arrestins can sequester MAPK but a specific role for arrestin-dependent sequestration of MAPKs in chemotaxis has not been identified. Arrestins are versatile, regulators of receptor desensitisation, trafficking and signalling through their ability to interact with numerous binding components (figure 1.4) [193], but there is still a number of unresolved questions regarding arrestins' functions that needs to be addressed. It is crucial to determine the different biological roles of GPCR-arrestin conformations and how those are regulated via other signalling mechanisms.

1.6.3. Downstream effector: Mitogen Activated Protein Kinases (MAPK)

Mitogen activated protein kinases (MAPKs) are highly conserved family of serine/threonine protein kinases involved in a variety of important cellular processes [194]. MAPK networks are important for the transmission of extracellular signals into

appropriate intracellular responses. They are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular responses such as growth, proliferation, differentiation, migration and apoptosis [195,196]. Therefore, dysregulation of theses cascades may be involved in the induction and progression of diseases such as cancer, diabetes, autoimmune diseases and developmental abnormalities [197–199].

MAPK pathways are comprised of a three-kinase core modules consisting of a MAPK kinase (MAPKKK or MAP3K) that phosphorylates and activates MAPK kinase (MAP2K, MEK or MKK) that in turn phosphorylates and dramatically increases the activity of one or more MAPKs [200]. Conventional MAPKs include the ERK1/2, the JNK1-3, the p38 isoforms (p38α, β, γ, and δ) and ERK5 [200,201]. A broad range of extracellular triggers including mitogens, cytokines, growth factors and environmental stressors stimulate the activation of one or more MAPKKKs via the receptor-dependent or -independent mechanisms [194]. While most MAPKKK, MEK and MAPKs display a strong preference for one set of substrates, there is significant cross-talk in a stimulus and cell-type dependent manner [201,202].

Abnormalities in the MAPK signalling are important in the development and progression of cancer [203–208]. Many MAPK pathways participate in stress signalling, and generally, stress comes in many forms, including hypoxia, detachment from substrate, inflammation and metabolic stress, and thus, large input of the network is important for sensing and processing stress signals [195]. This shows that there is an emergence in importance for stress-activated kinases in cancer, inflammation, DNA damage response and apoptosis [202,204].

ERK1/2 cascade is the best studied and first MAPK pathway elucidated which is dysregulated in approximately one-third of all human cancers [195,209]. In the ERK/MAPK pathway, ERK1/2 is activated upon phosphorylation by MEK1/2, which is activated when phosphorylated by activator, Raf (Raf-1, B-Raf and A-Raf) (figure 1.5) [195,210].

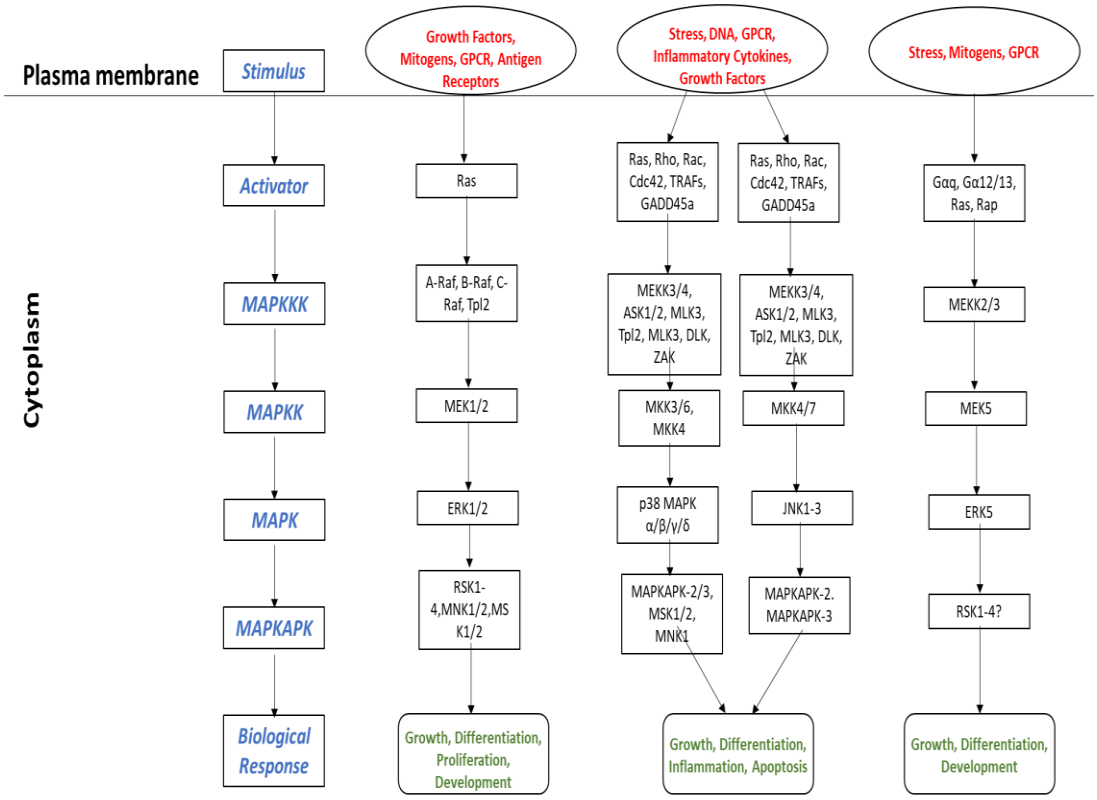


Figure 1.5 (Adapted from CST Signalling Technology 'MAP Kinase Signalling Resources'): An overview of MAPK signalling. Schematic representation of the MAPK cascades showing a range of extracellular triggers leading to the phosphorylation of the three core kinases and consequently result in signalling of cellular processes including growth, proliferation, differentiation, motility and apoptosis [195,210].

ERK/MAPK pathway is activated by a wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases (TKs), integrins and ion channels and this pathway is activated by GPCRs using different set of adaptors and binding proteins including, Ras, Rap1 (GEF proteins), Raf (MAPKKK), MEK1/2 (MAPKK) and ERK (MAPK) as some examples (figure 1.5) [210,211]. Activated ERK can regulate targets in the cytosol and also translocate to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression [195].

Studies into the mechanisms of oncogenic B-Raf signalling, a member of the Raf kinase family of growth signal transduction protein kinases, have highlighted novel mechanisms by which Raf kinases activate MEK/ERK signalling which differs from the classical Ras pathway [212]. B-Raf isoform is mutated at a high frequency in human

cancer, and mutation of V600E drastically elevates B-Raf kinase activity and its ability to activate the ERK pathway [212,213], but this does not apply to all cases.

Wan *et al.* (2004) studies showed that a few mutations do not elevate B-Raf kinase activity but they are able to activate MEK/ERK signalling [214]. This shows the specificity of the signalling and also, led to the discoveries that B-Raf heterodimerizes with Raf-1 and can signal through Raf-1 [213,215]. This indicates intricate complexities in MAPK signalling network, and hence, huge gaps of knowledge in this area to be filled.

Activated Raf activates MEK1/2 by phosphorylating serine 218 and 222 in the activation loop, and they are activated by specific Raf isoforms [216,217]. Activated ERKs phosphorylate numerous cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal proteins, and these signalling depends on the particular cell type with high specificity and reliability [218,219]. These factors may be linked to temporal differences in the strength and localisation of ERK within the cell [220].

Accumulating evidence have suggested that affecting ERK pathway can be a potential therapeutic drug target for cancer development and progression, particularly Ras, Raf and MEK [203,207]. PD98059 is a potent MEK inhibitor (IC_{50} = 2-7 μM), and it acts by binding to the inactivated form of MEK, and therefore preventing its phosphorylation by cRAF or MEK kinase [221]. It inhibits cell growth and proliferation in acute myelogenous leukaemia (AML) cell lines [222]. Activating ERK pathway not only play a role in cell proliferation, but it also potentially plays key roles in angiogenesis, cell migration, invasion and metastasis [223,224]. A study by Sharma *et al.* (2003) suggested that there is evidence of cross talk activation between MAPKs, in particular between ERK1/2 and p38, in co-ordinating cellular migration and proliferation in epithelial corneal wound healing and possibly general wound healing [225].

p38 MAPKs are members of the MAPK family that are activated by a variety of environmental stresses and inflammatory cytokines. p38 is required for the expression of tumour necrosis factor alpha (TNFα) and interleukin-1 (IL-1) during inflammatory responses, and characterisation of the function of p38 has been facilitated by using

SB203580, an anti-inflammatory drug that is a p38 inhibitor [226,227]. The p38 MAPK signalling pathway allows cells to interpret a wide range of external signals and respond appropriately by generating a plethora of different biological effects, and this diversity and specificity in cellular outcomes depends on the activation and regulation of core kinases, cross talk between signalling pathways and the nature of p38 MAPK substrates [228–230]. In addition to modulating cell survival, p38 MAPK is an interesting target to study tumour metastasis as there are literature supporting that p38 MAPK plays an essential role in modulation of cell migration and invasion [202].

SB203580 is a selective p38 MAPK inhibitor with IC_{50} values of 50 and 500 nM for p38 and p38β2 respectively [231]. SB203580 is a pyridimyl imidazole compound that is widely used due to its specificity in binding p38α and p38β but not p38δ and p38γ [232]. A great interest has developed in p38 inhibitors as they have been shown to have significant therapeutic benefits in inflammation such as arthritis, gastritis and pulmonary inflammation [233–235]. The recruitment process is a cascade of events, and hence intervening at one step will inevitably affect downstream events. This creates possibilities to investigate p38 MAPK signalling cascades, which may result in subsequent signalling events such as chemotaxis, using p38 inhibitors. Cara *et al.* (2001) suggested that there is a role of p38 MAPK in neutrophil chemotaxis and that p38 may be involved in the regulation of actin assembly [236]. Chemokines induce morphological changes in leukocytes that include rearrangement of cytoskeleton, to execute chemotaxis and there are literature showing the p38 inhibition may cause significant reduction effects upon downstream events of neutrophil emigration and chemotaxis [237–239].

Another study [240] suggested that p38 MAPK is a key signalling molecule for Ras, particularly H-Ras, induced cell motility. Ras is a protein superfamily of small GTPases and its expression has been suggested as a marker for tumour aggressiveness of breast cancer, including degrees of invasion [241,242]. Cancerous mutations in MAPK pathways have been shown to frequently affect Ras and B-Raf in the extracellular signal regulated kinase pathway [195]. The specific function of p38 MAPK depends on cell type, stimuli and also the isoform that is activated. The p38 MAPK pathway is usually activated in response to diverse stimuli and mediated by components upstream of p38, and in this case, it is H-Ras [202,240].

As discussed previously, there is emerging evidence that show expanding roles of arrestins as scaffolds and adapters in GPCR signalling and trafficking and one of the signalling pathways that falls into this category is MAPK pathway which involves interaction with molecules such as Src, Raf, ERK, apoptosis signal-regulating kinase 1 (ASK1) and JNK3 [180,183]. Arrestins function as MAPK scaffolds, bringing three components of MAPK signalling modules, such as JNK3, MKK4 and ASK1 (figure 1.6) [243]. All four arrestins were found to interact with these MAPK components but only arrestin 3 was needed for JNK3 phosphorylation, and interestingly, phosphorylated, active JNK3 can only be detected in association with arrestin 3 and is excluded from the nucleus [179,243]. These findings show that arrestin-activated kinase is only found in the cytoplasm [244].

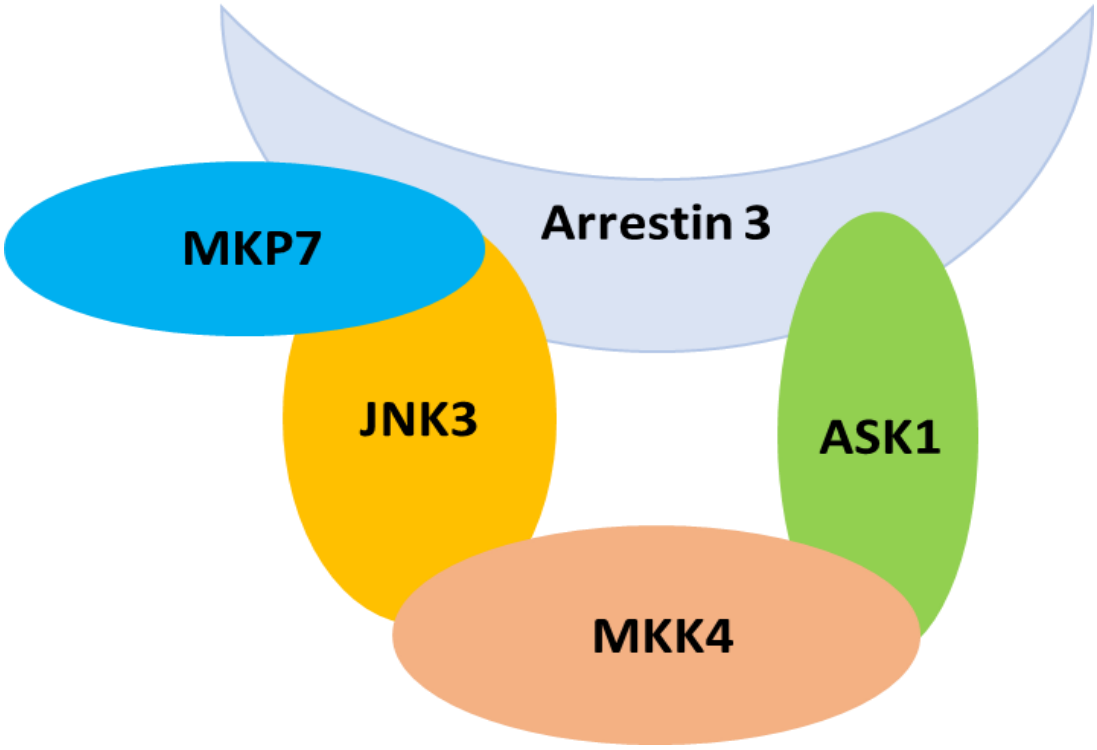


Figure 1.6 (Adapted from De Wire et al. (2007) [179]): Arrestins scaffolds for signalling. The arrestin scaffold for c-Jun N-terminal kinase (JNK) 3 activation. JNK3 and apoptosis signalling kinase 1 (ASK1) bind to arrestin 3 directly, whereas MAPK kinase 4 (MKK4) binds indirectly. Willoughby *et al.* (2005) recently showed that there is a possibility of fourth partner in this signalling module, MAP kinase phosphatase 7 (MKP7) and seem to be a negative regulator in an arrestin signalling module [245].

Metastatic cancer occurs through the orchestrated proliferation, migration, adhesion and differentiation of cells and MAPKs have been suggested to play a role in the

development and progression of cancer [202,229,240].The role of MAPKs in cancer is as pleiotropic as cancer itself, and there are studies that demonstrate the involvement of MAPKs in migration of cells [205,236] but the downstream signalling cascades after MAPK phosphorylation are still poorly understood.

1.7. Actin remodelling in migration of cells

Chemotaxis is a directed movement of a cell toward a chemical signal, and it requires a chemoattractant, which interacts with a cell surface receptor, to coordinate cell polarisation and motility in the direction towards the higher chemoattractant ligand concentration gradient [246]. Chemotactic signals then implicate in rearrangement of the actin cytoskeleton, leading to the formation of a leading edge, de-adhesion of the trailing edge and subsequent migration toward the chemoattractant [32,33]. Cell migration is a highly integrated and intricate multistep process which contributes to tissue repair and regeneration, and also drives disease progression in cancer [32,63]. Chemotaxis requires polarization of the cell such that it is actively moving in one direction the cell must effectively be sending opposing signals to each pole [34,247].

Actin assembly events involve nucleation, uncapping, severing and bundling of filaments, and for the cell to move along a substrate, there must be a certain amount of traction exerted, which is accomplished through the formation and disassembly of focal contacts at the leading edge and contractile forces of myosins bound to actin filaments [248,249]. The proteins that regulate all these events must be tightly controlled, including arrestins that are involved in spatial regulation of actin assembly and bundling proteins [81].

Actin cytoskeletal reorganisation and cell migration requires arrestins to act downstream of numerous receptors, which led to a great deal of interest in the mechanism by which arrestins can regulate these processes [81]. In addition to their classical, canonical role of arrestins as signal terminators in GPCR signalling, arrestins also act as signalling scaffolds, helping to localise molecules involved in cytoskeletal reorganisation and trigger cellular responses resulting in migration and chemotaxis as mentioned previously [81,179].

Cancer cells and immune cells both migrate toward a chemoattractant and upon binding of the ligand to its chemokine receptor, this would consequently lead to downstream signalling events, resulting in migration of cells. Arrestins have been hypothesised to regulate receptor turnover and hence, affect actin assembly. At the high end of chemokine concentration gradient, the cell will be surrounded and thus experiencing a uniform concentration of chemokine. At these high concentrations, arrestin mediated desensitisation and internalisation aid in receptor turnover [81]. As the cell moves along a gradient, the concentration of chemokine ligands gets lower and only receptors closest to the gradient will then be activated. At these concentrations, arrestin mediated actin assembly would then dominate, allowing the cell to move toward the source of the chemoattractant [18]. As the concentration of chemokine increases, cells may stop migrating due to loss of the ability to elaborate extensions or when they reach a region of uniform attractant, causing a loss of directional cue for the cell to migrate towards [250].

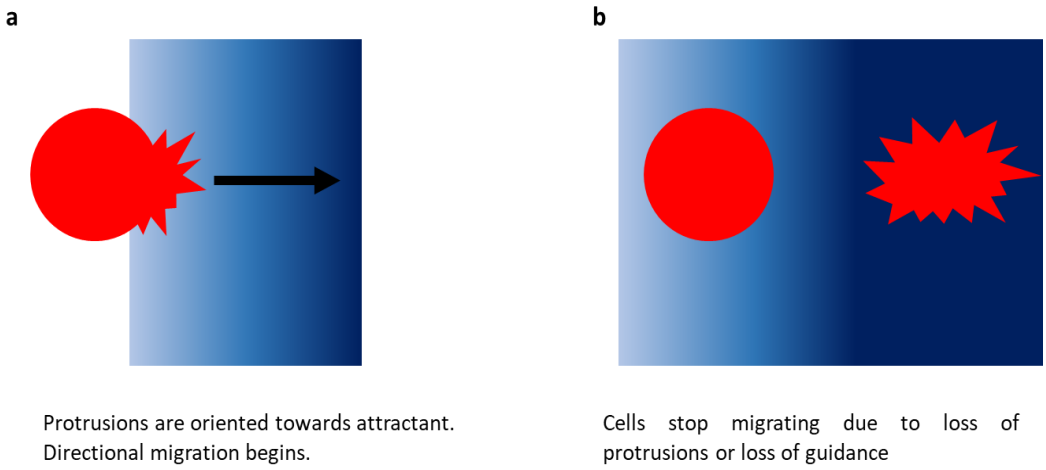


Figure 1.7 (Adapted from Aman et al. (2009) [250]): Cell migration towards chemoattractant. (a) Cellular extensions are polarized in the direction of migration in response to a gradient chemoattractant, usually chemokine or growth factor ligands (blue). (b) Cells stop migrating when they lose the ability to elaborate extensions (left) or they reach a region of uniform attractant (right).

PAR2 and AT1AR are two GPCR examples that have been demonstrated to promote the formation of arrestin scaffolds containing key actin assembly proteins [174,180]. These studies support a model where active arrestin signalling is involved in chemotaxis and showing actin assembly within a cell is the driving force behind directed cell movement

and can be regulated, both directly and indirectly, by various proteins [251]. Scott *et al.* (2006) have also identified that actin-bundling protein filamin as a novel arrestin binding partner, which suggest multifaceted roles for arrestins in actin assembly and cytoskeletal reorganisation and ultimately leads to migration of cells [251]. Additionally, proteins that are commonly associated with actin assembly include PI3K, RhoA, cofilin and these components can also be regulated by arrestins [170,174,186].

Various studies have suggested that arrestin-dependent ERK1/2 activation is important for actin reorganisation and/or chemotaxis, and the role of filamin in filapodia formation is well established [251,252]. There are also studies suggesting that myosin binds actin filaments at the back of migrating cells, stabilising polarity by inhibiting protrusion formation while at the leading edge, myosin is important for sensing changes. They can also help in promoting traction, through contractile forces exerted on the extracellular matrix and helping the cell moves along its substrate [253–256].

The identification of actin-binding and regulatory proteins as arrestins-binding partners suggests that arrestins scaffolding function is important for actin reorganisation, and ultimately resulting in cell migration. Research into the molecular basis of cell migration has progressed rapidly over the past few years, and even though key regulatory molecules have been identified, mechanisms of how cells migrate are still poorly established, for example, how cells establish and maintain their polarity and how cells recognise their targets [32]. Emerging evidence showing the involvement of arrestins in actin cytoskeleton reorganisation complicates the studying of spatially segregated component processes across the cell [18,81]. There are so many gaps of knowledge regarding this area that is yet to be explored to further understand the molecular mechanisms of migration of cells.

1.8. Concluding remarks

The dysregulation of migration is causative to numerous diseases and understanding why this is happening is critical for the identification of potential therapeutic targets for these diseases. The chemokine receptor CXCR4 is directly linked to inflammatory and metastatic diseases, but it is also widely characterised intra- and extracellularly, together with CCR5, due to its involvement as a co-receptor in HIV viral entry [15,257].

This makes CXCR4 a particularly good model system in which the specifics of cell migration can be determined with numerous cell lines available which endogenously express CXCR4 (Jurkat, MCF-7 cells). Even though there is a large amount of research which has been undertaken over the last decade to establish signalling events governing pathways downstream of activated CXCR4, there are still so many unresolved areas to be explored.

There is evidence, as mentioned previously, that support that PKC phosphorylation, other than GRK2 phosphorylation, triggers signalling events upon chemokine receptor activation, but whether they directly affect migration of cells is still unknown. There are a large number of diverse inhibitors available for use to study the downstream signalling upon CXCR4/CXCL12 activation. Investigation of CXCR4 signalling using cell permeable small molecule inhibitors enables us to study and identify potential targets and to map out the possible pathways that may lead to migration of cells. The inhibitors can be used to clarify cell specific type from receptor specific type events and to identify targets which may have an effect in migration of cells.

The other proteins of interest are arrestins, which are known to be involved in cell migration via chemokines/chemokine receptors signalling but the molecular mechanisms of this signalling event have not been well characterised. Knockdown and overexpression of proteins in this case can reveal potential drug targets and to further understand the role of these proteins in downstream signalling cascades, resulting in migration of cells.

Studying migration of cells is important because in cancer, metastatic tumours have poor prognosis, as they are usually difficult to treat with current therapies. Understanding how chemokine/chemokine receptor influence cells to migrate and how tumour metastasize would create possibilities to beneficial therapeutic targets and help combat different diseases. This thesis will outline interactions of proteins of interest in CXCL12-induced signalling events upon CXCR4 activation.

1.9. Research objectives

Pharmacological disruption of chemokine receptor mediated processes represents a path to therapeutic intervention in many diseases. Even though chemokines and their receptors have been of interest for over a decade, there is still lack of understanding of chemokine receptor mediated signalling. CXCR4 signalling events are still poorly understood due to the large number of protein interactions which may potentially be involved.

The introduction demonstrated the possible proteins that may be involved in CXCR4 signalling event, which may lead to migration of cells, such as PKC and arrestins. PKC and arrestins are involved in chemokine/chemokine receptor signalling event but the relationship between them are still not clearly defined. Identifying if these events occur simultaneously and/or are dependent on the specifics of the chemokine receptor signalling, for example, if they are receptor or cell type specific, is critical. By using small molecule inhibitors to disrupt protein signalling and comparing effects in downstream signalling cascades, this may give understanding of chemokine receptor signalling and the potential to identify novel receptor specific targets for disruption of chemokine receptor function.

In order to achieve this, the experimentation that was undertaken included:

CHAPTER 3: Chemokines have redundant actions on target cells and promiscuous receptor usage [258]. The interaction of chemokines with their receptors is characterised by considerable promiscuity, however, some groups have found different receptor signalling and trafficking responses to individual chemokines, suggesting that there may be biased signalling [259,260].

Hypothesis: We hypothesised that different chemokines illicit different signalling pathway responses in different cell types.

Research undertaken: Characterisation of CXCR4 by investigation into the chemotactic responses stimulated by various chemokines which would result in

signalling events in suspension leukemic T-lymphocytes Jurkat cells and adherent breast cancer MCF-7 cells.

CHAPTER 4: Woerner *et al.* (2005) showed that there was increased phosphorylation of Ser339 following SDF stimulation and that increased phosphorylation of Ser339 was also observed following phorbol ester treatment [143], suggesting that this may be a potential PKC phosphorylation site of CXCR4.

Hypothesis: We hypothesised that PKC phosphorylation contributes to CXCL12-induced migration in different cell types.

Research undertaken: Investigation and determination of the role of PKC in CXCL12-induced migration in Jurkat and MCF-7 cells to see if there is a difference in effects in different cancer cell lines. Comparison of effects of PKC inhibition in different chemokine receptors and different cell lines – to see whether they are cell-type or receptor-type specific.

CHAPTER 5: Over the past few years, it has been increasingly appreciated that arrestins not only play a role in desensitisation of activated GPCRs but they have also emerged as multifunctional adaptor/scaffold proteins that dynamically assemble a wide range of multiprotein complexes in response to stimulation of most 7TMRs [261]. Arrestins are not only required for the desensitisation of several chemokine receptors, including CXCR4, but they have been found to help orchestrate the cellular signals to chemotactic signals [14,18,144].

Hypothesis: We hypothesised that arrestins is important in CXCL12-induced migration in different cell types.

Research undertaken: Exploring the role of arrestins in CXCR4/CXCL12 signalling axis as they have been shown to be essential regulators in chemotaxis, but the mechanisms of their involvement in this particular signalling axis are still not yet established. Here, knockdown and overexpression of arrestins by using DNA plasmids were undertaken to confirm the role of arrestins in cell signalling events, including

migration, of Jurkat and MCF-7 cells in relation to CXCL12-induced migration via CXCR4 chemokine receptor.

CHAPTER 2: Materials and methods

2.1. Cell lines and tissue culture

2.1.1. Acute monocytic leukaemia cell line THP-1

THP-1 cells were originally obtained from the peripheral blood of a one-year old male donor with acute monocytic leukaemia [262]. THP-1 cells have been shown to be good study models as they endogenously express a number of human chemokine receptors. THP-1 cells were obtained from American Type Culture Collection (ATCC) (Teddington, UK) and were cultured using RPMI (Corning, Biosera) supplemented with 10% v/v Foetal Calf Serum (FCS) (Invitrogen), 100 µM non-essential amino acids (Gibco) and 2 mM glutamine (Invitrogen).

2.1.2. Leukemic T-cell lymphoblasts Jurkat

Jurkat cells are T lymphocytes originally derived from one-year old male with acute T cell leukaemia [263]. They have been proven to be useful and good model systems to study as they express various chemokine receptors susceptible to viral entry, particularly HIV. This cell line is a suitable transfection host, and it has high expression of CXCR4. Jurkat cells were obtained from American Type Culture Collection (ATCC) (Teddington, UK) and were cultured using RPMI (Corning, Biosera) supplemented with 10% v/v FCS (Invitrogen), 100 µM non-essential amino acids (Gibco) and 2 mM glutamine (Invitrogen).

2.1.3. CHO.CCR5 stably transfected Chinese Hamster Ovary fibroblasts

Chinese Hamster Ovary (CHO) cells have proven to be easy to culture and are relatively large in size, which makes them good specimens for microscopy. CHO.CCR5 cells were acquired from Professor J. McKeating (Nuffield Department of Medicine, Oxford). CHO cells were transfected with pcDNA3 encoding Human CCR5 and selected for stable expression in 10% FCS DMEM (Corning, Biosera) with 2 mM glutamine in the presence of selective aminoglycoside antibiotic G418 (Invitrogen) (400 µg/mL).

CHO.CCR5 cells were cultured in complete DMEM (10 % FCS, 100 μM non-essential amino acids and 2 mM glutamine) supplemented with 400 μg/ mL G418 (Invitrogen) to ensure continued expression of CCR5.

2.1.4. Human breast adenocarcinoma cell line MCF-7

MCF-7 cells are a widely studied epithelial cancer cell line derived from breast adenocarcinoma [264]. It is the best characterised and most widely use of all the human breast cancer cell lines. MCF-7 cells can be used to study various pathways that are involved in cell migration, including PI3K and MAPK involvement. MCF-7 cells were cultured in DMEM (Corning, Biosera) supplemented with 10% v/v FCS (Invitrogen), 100 μM non-essential amino acids (Gibco) and 2 mM glutamine (Invitrogen).

2.1.5. Routine tissue culture procedures for cell lines

Both adherent and suspension cell lines were cultured in 75 cm^2 flasks (Corning) in a humidified atmosphere at 37°C with 5% CO_2. At 80-95% confluence, adherent cells were removed from the flask by adding PBS (1.5 mM potassium phosphate monobasic, 3 mM potassium phosphate dibasic, 150 mM NaCl; pH 7.2) supplemented with 2.5 mM EDTA and incubating them for 5 – 10 minutes at 37°C with 5% CO_2. Flasks were then gently agitated to suspend cells. Cells were centrifuged at 1800g for 5 minutes and resuspended in relevant growth medium after which cell density was determined microscopically with the use of a haemocytometer. Cells were then used for experimentation or passaged by reducing cell density by 80-90% and continued culturing. The suspension cell line, THP-1, was cultured for 3 days or until cell density reached 6 X 10^4/ mL at which point cell density was reduced to 2 X 10^4/ mL by removal of suspended cells and addition of fresh media. Jurkat cells was cultured for 2 days or until cell density reached 1 X 10^6/ mL at which point cell density was reduced to 1 X 10^5/ mL by removal of suspended cells and addition of fresh RPMI. Where cells were to be cryopreserved, 1 X 10^6 cells were centrifuged, resuspended into 1 mL of 10% v/v DMSO in FCS and transferred into cryotubes. To prevent damage by freezing these cryotubes were first wrapped in tissue and chilled to -80°C for at least 24 hours before long term storage in liquid nitrogen at -196°C.

2.2. Small molecule and oligomeric inhibitors

A complete list of the small molecule and protein inhibitors is detailed below.

Table 2.1. PKC inhibitors

Inhibitor	Supplier	Stock Conc/ Vehicle	Working Conc
GF109203X	Tocris	23.5 mM DMSO	5 μM
Staurosporine	Tocris	100 μM water	10 nM
Rottlerin	Tocris	2 mM ethanol	4 μM
CID755673	Tocris	4.6 mM ethanol	11 μM

Table 2.2. Kinase inhibitors

Inhibitor	Supplier	Stock Conc/ Vehicle	Working Conc
PD98059	Abcam	25 mM DMSO	25 μM
SB203580	Tocris	25 mM DMSO	10 μM

2.3. Chemokine ligands

A complete list of the chemokine ligands used is detailed below.

Table 2.3. Chemokine ligands used for experiments; CF- calcium flux, CTX – chemotaxis assays, IF- immunofluorescence, FC- flow cytometry, WHA – wound healing assays. Note: All chemokine ligands stock was made up in purified water

Chemokines	Supplier	Chemokine receptor	Concentrations used
CCL1 (I-309)	PeproTech	CCR8	10 nM CTX
CCL2 (MCP1)	PeproTech	CCR2	0.01 – 100 nM CTX
CCL3 (MIP-1α, isoform D26A)	CCL3 (2-70) - a gift from L. Czaplewski (British Biotech) [265]	CCR1, CCR5	100 nM CF 1 nM CTX 100 nM IF
CCL5 (RANTES)	PeproTech	CCR1, CCR3, CCR5	0.01 – 100 nM CTX
CCL8 (MCP2)	PeproTech	CCR2, CCR5	1 nM CTX
CCL23 (MIP-3)	PeproTech	CCR1	10 nM CTX
CXCL8 (IL-8)	A gift from K. Schmitz (TU Darmstadt) [266]	CXCR1, CXCR2	50 nM CF
CXCL11 (IP-9)	PeproTech	CXCR3	0.01 – 100 nM CTX
CXCL12 (SDF1-α)	PeproTech	CXCR4, ACKR3	10 nM CF 0.001 – 10 nM CTX 10 nM IF 15 nM FC 10 nM WHA
CXCL12 N-His Tag (SDF1, isoform a)	A gift from K. Schmitz (TU Darmstadt)	CXCR4	1 – 500 nM CF 0.01 – 500 nM CTX
CXCL14 (BRAK)	PeproTech	Orphan receptor	0.001 – 300 nM CTX

2.4. Antibodies

A complete list of primary and secondary antibodies is detailed below.

Table 2.4. Primary and secondary antibodies used for experiments; IF -Immunofluorescence, FC- Flow Cytometry, WB- Western Blot

Primary Antibody	**Supplier**	**Assay use**	**Dilution factor**
HEK/1/85a/7a rat monoclonal	A gift from J.A. McKeating (Oxford)	IF	1:100
CXCR4 (125G5): sc-12764	Santa Cruz Biotechnology	IF	1:100
Human CXCR4 Antibody	R&D systems	FC	1:2000
β-Arrestin-1/2 Antibody (H-290): sc-28869	Santa Cruz Biotechnology	WB	1:100-1:1000
β-Arrestin-1/2 Antibody (21-B1): sc-53781	Santa Cruz Biotechnology	WB	1:100 – 1:1000
β-Arrestin-1/2 (A-1): sc-74591	Santa Cruz Biotechnology	WB	1:500
β-Actin (C4): sc-47778	Santa Cruz Biotechnology	WB	1:1000
Secondary Antibody	**Supplier**	**Assay use**	**Dilution factor**
Goat anti-mouse FITC	Sigma- Aldrich	IF	1:1000
Goat anti-mouse TRITC	Sigma- Aldrich	IF	1:1000
Goat anti-mouse IgG (H+L), F(ab')2 fragment, CF 647	Sigma- Aldrich	FC	1:200
Anti-mouse IgG horseradish peroxidase	Sigma- Aldrich	WB	1:5000

2.5. Plasmid DNA

The pEGFP arrestin 2/3 plasmids were produced by cloning relevant arrestin DNA into *HindIII/ ApaI* digested pEGFP-N1 plasmids (Clontech, Saint-Germain-en-Laye, France) as described and validated and were a gift from E. Kelly (Bristol). In addition to pEGFP arrestin 2 and pEGFP arrestin 3 plasmids, arrestin 2 mutant (arrestin 2-V53D) plasmid was made by point mutation, where the valine residue was substituted for an aspartic acid as described [267]. pEGFP.C2 plasmids (Clontech) were used as mock transfection controls.

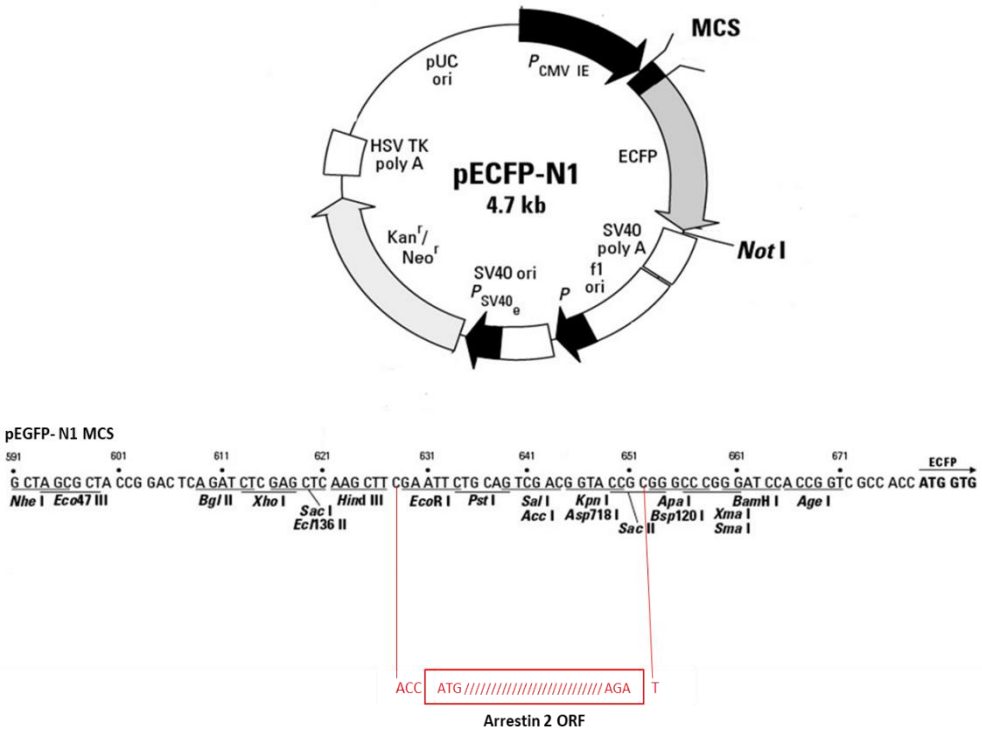


Figure 2.1: Mammalian arrestin 2 amplified by PCR and cloned into *HindIII/ApaI* digested pEGFP-N1 (Clontech). DNA plasmids were gift from E. Kelly (Bristol). Arrestin 2 insert can be excised by *HindIII/ ApaI* digestion. EGFP is on the C-terminal end of arrestin 2 [268].

The CXCR4 coding sequences without a stop codon were amplified using sense and antisense primers harbouring unique *HindIII* and *BamHI* sites. The fragments were then subcloned in frame into *HindIII*/*BamHI* sites of a humanized form of *Renilla Luciferase* (hRluc)-N3 vector as described and validated [269]. The arrestin3/GFP10 construct was prepared from the vector pcDNA3.1-GFP10. CXCR4-RLuc3 and h-arrestin3-GFP10 plasmids were kindly provided by N. Heveker (Montreal).

2.5.1. Preparation of plasmid DNA from bacterial colonies

Chemically competent *E. coli DH5Af*[1] (Invitrogen) were transformed with the required plasmid DNA to allow amplification of plasmids. 50 µL of *E. coli DH5Af*[1] were thawed in iced-water and gently broken down with a pipette to form homogenous slurry. 1-5 µg of the relevant plasmid DNA construct was added to the *E. coli* and incubated for 30 minutes in the iced-water bath. Transformation was then induced by heat shocking the bacteria at 42°C for 90 seconds before 2 minutes incubation in iced-water. 1 mL of pre-warmed Lennox Broth (LB) was added to the heat shocked *E. coli* before incubation in rotary shaker at 37°C, 200 rpm for 2 hours. 25 µL of the resulting bacterial broth was spread on prepared LB agar plates containing kanamycin (ThermoFisher Scientific) (100 µg/ mL) or ampicillin (ThermoFisher Scientific) (100 µg/ mL) and grown, upside-down, overnight at 37°C. Viable colonies were picked then incubated overnight in 20 mL LB containing kanamycin (ThermoFisher Scientific) (100 µg/ mL) or ampicillin (ThermoFisher Scientific) (100 µg/ mL). The cultured bacterial broth was then frozen down at -80°C or used for plasmid amplification described below.

2.5.2. Amplification of plasmid DNA

Plasmid DNA was purified using the Qiagen MIDI kit (Qiagen). The relevant transformed *DH5Af*[1] strain was grown overnight and the bacterial broth was centrifuged at 6000g for 10 minutes. The supernatant was removed and 4 mL of P1 buffer containing RNAse was added to resuspend the pellet. 4 mL of P2 buffer was added to the suspension then agitated sufficiently to turn the solution a uniform blue before incubation for 5 minutes at room temperature. 4 mL of P3 buffer was added to precipitate protein then the solution was centrifuged at 6000g for 10 minutes. Supernatant was then filtered through an activated Qiagen column and flushed with 10 mL QC wash buffer. Plasmid DNA was then eluted from the column with 5 mL QF elution buffer. 3.5 mL of propan-2-ol was added to the eluent to precipitate DNA during centrifugation for 1 hour at 6000g. The supernatant was removed, and the pellet washed in ethanol before being dried and dissolved in 500 µL deionised water. Plasmid DNA purity was determined by gel electrophoresis after digestion with *HindIII*/ *EcoRI* restriction enzymes (Promega). Plasmid DNA concentration was determined by Nanodrop spectrometer system (ThermoFisher Scientific).

2.6. Electroporation transfection

2.6.1. Electroporation transfection of plasmid DNA

Cells were counted and centrifuged to give 3 X 10^6 cells per tube. Cells were resuspended in 250 µL HEPES buffered electroporation solution (HEPES 20 mM, NaCl 137 mM, KCl 5 mM, Dextrose 6 mM, Na_2HPO_4 0.7 mM, pH 7.5) to which 5 µL t-RNA (Sigma Aldrich) and 2 µg plasmid DNA was added in 0.2 cm transfection cuvette (Sigma Aldrich) before electroporated, using AMAXA Nucleofector 2b (Lonza), at the relevant setting (X-005 for Jurkat cells). 500 µL of simple RPMI was added to the mixture in the cuvette and the contents were incubated at room temperature for 10 minutes. The solution was removed with a pipette and transferred to pre-incubated 9.5 mL T-25 flasks (Corning) containing relevant growth media. Cells were incubated for 24 hours before assessment of transfection by flow cytometry.

2.6.2 Electroporation transfection of siRNA

Scrambled siRNA (Dharmacon) was used as a negative control and it has the same nucleotide composition but not the same sequence as the test siRNA. Scrambled siRNA is used to distinguish sequence-specific silencing from non-specific effects. Scrambled siRNA can be used as a negative control as it is designed to not target any known gene in the cell. Stock concentration of 2 µM scrambled siRNA diluted in 1X siRNA suspension buffer was made up and used in working concentrations of 50 nM in transfection assay. 5 nmol ARRB1 and ARRB2 ON-TARGETplus siRNA reagents (Dharmacon) were prepared using Dharmacon siRNA resuspension protocol as stock concentration of 20 µM in 250 µL 1x RNAase free siRNA buffer (Qiagen). Concentration of siRNA was verified using the Nanodrop spectrometer system (ThermoFisher Scientific) and 1 in 100 dilution aliquots were prepared to limit the number of freeze-thaw cycles. Cells were counted and centrifuged to give 3 X 10^6 cells per tube. Jurkat cells were resuspended in 200 µL HEPES buffered electroporation solution (HEPES 20 mM, NaCl 137 mM, KCl 5 mM, Dextrose 6 mM, Na_2HPO_4 0.7 mM, pH 7.5) to which 50 nM siRNA reagents (50 nM scrambled siRNA as negative control) was added into each respective 0.2 cm transfection cuvettes (Sigma Aldrich) before electroporated at the relevant setting (X-005 for Jurkat cells). 500 µL of simple RPMI was added to the

mixture in the cuvette and the contents are incubated at room temperature for 10 minutes. The solution was removed with a pipette and transferred to pre-incubated 9.5 mL T-25 flasks (Corning) containing relevant growth media. Cells were incubated for 48 hours before assessment of transfection and experimental procedures.

2.7. Chemical transfection

2.7.1. Chemical transfection of plasmid DNA

1 X 10^5 MCF-7 cells were seeded in 24-well plate in 1 mL of growth medium 24 hours before transfection to ensure optimal confluency of 70 – 90%. 2 µg of respective DNA was diluted in 100 µL of simple DMEM. 4 µL of Turbofect transfection reagent (ThermoFisher Scientific) was added to the diluted DNA and mixed immediately by pipetting or vortexing. Mixture was then incubated at room temperature for 20 minutes before 100 µL of the transfection reagent/ DNA mixture was added drop-wise to each well in the 24-well plate. The plate was then gently rocked to achieve even distribution of the complexes immediately before the plate was incubated at 37°C, 5% CO_2. The plate was then be analysed after 24 hours using an inverted Leica DMII fluorescence microscope with GFP filter cube to ensure effective transfection and to be used for different assays. In wound healing assays, transfection efficiency was measured by counting an area of cells in overlay of fluorescence images, taken with the GFP filter cube, in the bright-field images. Transfection efficiency was then quantified by fluorescence cells divided by total cells in the area and multiply by 100%. In calcium flux and transwell migration assays, transfection efficiency was gauged by measuring fluorescence at excitation/ emission of 485/520 nm using BMG Labtech Fluorostar Optima plate reader (BMG Labtech, Germany).

2.7.2. Chemical transfection of siRNA

Scrambled siRNA (Dharmacon) was used as a negative control and it has the same nucleotide composition but not the same sequence as the test siRNA. PKCα and PKCζ siRNA (Qiagen) were prepared and diluted to working concentrations in RNAase free water (Qiagen). Concentration of siRNA was verified using the Nanodrop spectrometer system (ThermoFisher Scientific). MCF-7 cells were counted and centrifuged to give 2

X 10^5 cells per well in 24-well plates. Cells were transfected using Lipofectamine RNAiMax reagent (ThermoFisher Scientific). Diluted 50 nM of PKCα and PKCζ siRNA (Qiagen) were added to dilute the Lipofectamine RNAiMax reagent (ThermoFisher Scientific) in a 1:1 ratio. Mixture was then incubated for 5 minutes at room temperature. The siRNA-lipid complex was then added to cells and cells were incubated for 48 hours before assessment of transfection and experimental procedures.

2.8. Calcium flux

Adherent cell lines were harvested by addition of PBS containing 2.5 mM EDTA. THP-1 cells were collected, centrifuged at 1800g for 5 minutes. All cells were re-suspended in calcium flux buffer (137 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES pH 7.4, 25 mM D-glucose). Cells were spun down and washed twice more in calcium flux buffer before resuspension in 1 mL of calcium flux buffer to give a concentration of 1 X 10^6 cells/mL. Cells were then treated with the relevant concentrations of inhibitor and loaded with the membrane permeable acetoxymethylester conjugated form of Fura-2 (Invitrogen). Fura-2 AM is a cell permeant, intracellular calcium indicator. Fura-2 AM ester is a non-polar calcium insensitive compound that freely crosses the cell membrane. Once inside the cell, ubiquitous cellular esterases hydrolyse the compound to a calcium ion sensitive negatively charged moiety, Fura-2. Due to its anionic charge, Fura-2 can no longer cross the cell membrane and is essentially trapped within the cell. Fura-2, upon binding to Ca^{2+}, exhibits a shift in its absorption or excitation peak from 338 nm to 366 nm.

2 μM of Fura-2 AM was added to all cell samples before incubation at 37°C, 5% CO_2 for 30 minutes. Cells were then centrifuged at 1800g and washed three times with 1 mL calcium flux buffer. 100 μL of cell was added to each well of a black, opaque reader plate (ThermoFisher Scientific) before calcium flux assessment by addition of different agonist injected directly into the wells using BMG Labtech Fluorostar Optima plate reader (BMG Labtech, Germany). Changes in calcium release were determined by ratiometric analysis of alteration in fluorescence sequentially stimulated by 340 nm and 380 nm light detected at a fixed emission frequency of 510 nm. Data were recorded and analysed using BMG Optima software and were represented as ratio of the fluorescence detected, which is directly related to the amount of intracellular calcium.

The change in fluorescence was calculated as the difference between the pre-stimulated 340 nm/ 380 nm ratio and the peak value after stimulation.

2.9. Migration assays

2.9.1. Chemotaxis migration assay using ChemoTX plate

Chemotaxis assays for Jurkat cells were carried out in ChemoTX 5 μm pore chemotaxis plates (Neuroprobe Inc, USA). Wells to be used in the chemotaxis plate were blocked with 30 μL blocking buffer (1% BSA in simple RPMI) for at least 30 minutes at room temperature. Chemokine solutions were prepared at 1 – 10 nM, depending on assay requirements, in working buffer (0.1% BSA in simple RPMI). Jurkat cells were micro-centrifuged at 5000g for 5 minutes, washed once in simple RPMI and resuspended in 39 μL working buffer to give 25 X 10^4 cells per well with two wells worth of cells per sample for stimulated and unstimulated runs. Relevant treatments or vehicle control were added before an incubation for 30 minutes at 37°C, 5% CO_2. The blocking buffer was removed from the plate and replaced with 31 μL of the relevant chemokine solution (working buffer for unstimulated samples). The membrane was attached and 20 μL of cell suspension was added onto the top surface of the membrane. The plate was then incubated in a fully humidified chamber for 5 hours before membrane was dried and removed. The cells in each well were then counted with a haemocytometer.

2.9.2. Transwell migration assay

Adherent cells were cultured until around 90% confluence was reached and detached by incubation with PBS with 2.5 mM EDTA for 5 minutes. Cells were washed in PBS and re-suspended in 12 mL growth medium of growth medium at a density of 3 X 10^5 cells per mL. Cells were pipetted into 6 well plates (2 mL per well) and incubated at 37°C, 5% CO_2 for 24 hours to allow adherence. Cells were then chemically transfected with relevant DNA plasmids (refer to section 2.7.1; page 56) and analysed using an inverted Leica DMII fluorescence microscope using GFP filter cube to see if there was effective transfection. Cells were then removed by incubation at 37°C, 5% CO_2 with PBS with 2.5 mM EDTA for 15 minutes until most cells were suspended in solution. In a sterile 24 well plate, 600 μL of 0.2% BSA/DMEM were added in each well and relevant

chemokine was added into positive wells. In these chemotaxis assays, 8 µm pore Greiner Bio-One inserts (PETMembrane) were used and inserted in each well. Cells were counted at a density of 1 X 10^6 per mL and washed gently with 5 mL PBS and re-suspended with 0.2% BSA/DMEM. 200 µL of cells were added in each insert and plate was incubated at 37°C, 5% CO_2 for 24 hours. Inserts were removed and added onto a new, sterile 24 well plate. Cells were then gently removed from inserts by incubating with 0.25% Trypsin EDTA (Gibco) at 37°C, 5% CO_2 for 30 minutes. 200 µL cells were added to each well of a black, opaque reader plate (ThermoFisher Scientific) before assessment at excitation/ emission of 485/520 nm using BMG Labtech Fluorostar Optima plate reader (BMG Labtech, Germany).

2.9.3. Wound healing assay

Sterile 12 well plates were prepared by marking the outside of the bottom surface with two parallel lines with indelible marker per well which forms the reference points for wound healing quantification. This procedure was done before the addition of cells. Adherent cells were cultured until around 90% confluence was reached and re-suspended by incubation with PBS with 2.5 mM EDTA for 5 minutes. Cells were washed in PBS and re-suspended in 24 mL of growth medium at a density of 3 X 10^5 cells per mL. Cells were pipetted into pre-marked 12 well plates (2 mL per well) and incubated at 37°C, 5% CO_2 for 24 hours to allow adherence and spreading of the cells. Scratches were made in the cell layer perpendicular to the reference lines using a 200 µL pipette tip drawn along the side of the plate lid. The growth medium was then removed, and the cells washed gently twice in PBS to remove loosened and suspended cells. 200 µL of simple DMEM was then placed in each well and the wounds were imaged in duplicate using an inverted Leica DMII fluorescence microscope in bright-field mode at 10X magnification in such a way that the horizontal reference line was visible in all images. The relevant chemokines and inhibitors were added into respective wells at working concentration (tables 2.1, 2.3) Images were then collected at 24 hours (timepoint 24) after addition of treatments. Wound healing was quantified using Microsoft Powerpoint by aligning the horizontal reference line to vertical and then aligning vertical guides to the midpoint of the cell which was closest to the centre of the scratch but part of the confluent monolayer. The perpendicular width between the two edges of the scratch could then be determined. For timepoint images, the

transformation setting used for the t=0 (timepoint 0) images were used, and perpendicular width re-determined as described (figure 2.1).

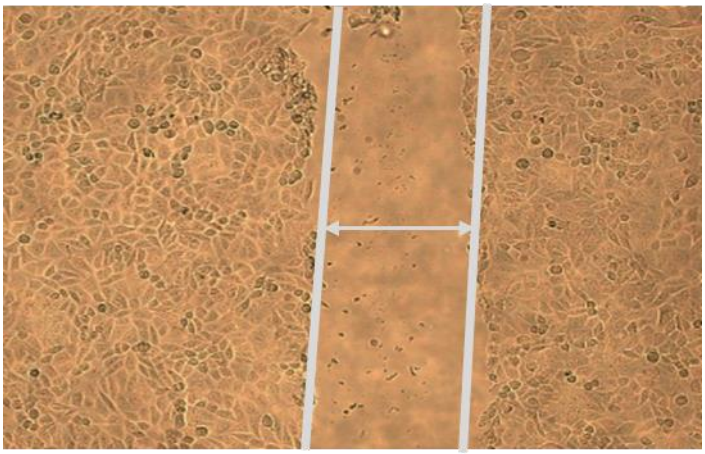


Figure 2.2: Image representative of wound healing assay on MCF-7 cells. A scratch was made on a cell monolayer and images were captured at timepoint 0, before addition of chemokine, and at timepoint 24 to assess migration of cells. The perpendicular width (arrowed line) between the two edges of the scratch were measured. Quantification of migration of cells into the wound was calculated as ratio of woundwidth at timepoint 24 to woundwidth at timepoint 0. A value of 1 denotes no migration occurred, whereas a value < 1 denotes cell migration.

2.10. Imaging techniques

2.10.1. Phalloidin actin stain

CHO.CCR5 or transfected MCF-7 cells (refer to section 2.7.1; page 56) were split at around 95% confluence with 4mL PBS with 2.5 mM EDTA and 200 µL of cells were placed over washed coverslips in a 12 well plate. Cells were incubated for 24 hours to allow cells adherence. Once 95% confluence was reached, the inhibitors were added at working concentrations and left for 30 minutes. Ligands were then added to relevant wells at working concentration (100 nM CCL3 and 10 nM CXCL12) and plates were incubated at 37°C, 5% CO_2 for 1 hour. Cells were washed twice in PBS and fixed by adding 300 µL 4% paraformaldehyde solution for 10 minutes. Cells were gently washed twice in PBS and then treated with 300 µL 0.1% Triton X-100 (FisherBioTech) solution for 5 minutes for cell permeabilization. Cells were washed twice in PBS and then 2 µL of Alexa Fluor 488 tagged phalloidin (Invitrogen) was added to 200 µL PBS or 300 µL of diluted Phalloidin CruzFluor 594 Conjugate (1 in 2000 in 1 % BSA/PBS) (Santa Cruz)

was placed onto the slides before incubation at 4°C in the dark for 30 minutes. Cells were gently washed twice with PBS and coverslips with cells were mounted onto glass slides with DPX mountant (ThermoFisher Scientific), which contains an anti-oxidant that inhibits stain fading. Cells were analysed immediately using an inverted Leica DMII fluorescence microscope with a filter setting suitable for the fluorophore used.

2.10.2. CCR5 receptor internalisation

Cells were seeded overnight onto coverslips. Once 95% confluence was achieved, the inhibitors were added to respective wells at working concentrations and plates were incubated at 37°C, 5% CO_2 for 30 minutes. Cells were then stimulated with 100 nM CCL3 and plates were incubated at 37°C, 5% CO_2 for 1 hour. Cells were gently washed once with PBS and HEK/1/85a/7a (1:100) in 1 mL simple DMEM (1 mL PBS as negative control) was placed onto the cells which were then incubated at 4°C for 1 hour. Cells were gently washed three times with PBS before fluorescently tagged secondary antibody (1:1000) was added in 1 mL PBS and incubated at 4°C for 1 hour. Cells were washed three times in PBS and coverslips were mounted onto glass slides with DPX mountant (ThermoFisher Scientific). Cells were analysed immediately using an inverted Leica DMII fluorescence microscope with a filter setting suitable for the fluorophore used.

2.10.3. CXCR4 receptor internalisation

Adherent cells were seeded overnight onto coverslips. Once 95% confluence was achieved, the inhibitors were added at working concentrations and incubated at 37°C, 5% CO_2 for 30 minutes. Cells were then stimulated with 10 nM CXCL12 and incubated at 37°C, 5% CO_2 for 1 hour. Cells were washed twice with PBS and stained with CXCR4 mAB 12G5 (Santa Cruz Biotechnology) (1:100) in 1 mL PBS (1 mL PBS as negative control) which were then incubated at 4°C for 1 hour. Cells were washed three times with PBS before fluorescently tagged secondary antibody (1:1000) was added in 1 mL PBS and incubated at 4°C for 1 hour. Cells were gently washed twice with PBS and coverslips were mounted onto glass slides with DPX mountant (ThermoFisher Scientific). Cells were analysed immediately by an inverted Leica DMII fluorescence microscope with a filter setting suitable for the fluorophore used.

For suspension cells' staining, the procedure above was followed although cells were prepared in suspension in 1.5 mL tubes with cell density of 5 X 10^6 per mL in PBS and centrifuged between treatments and washes. Mounting of the cells was achieved by resuspending pelleted cells in 50 µL of glycerol mounting mixture (90% glycerol in PBS) then pipetting 20 µL of the resulting suspension into a glass cover slip. These coverslips were dried at 4°C overnight and analysed using an inverted Leica DMII fluorescence microscope with a filter setting suitable for the fluorophore used.

2.10.4. Microscopy

Fluorescently stained slides and transfected cells with EGFP were imaged using Leica wide field fluorescence microscope fitted with 10, 40, 63X inverted air objectives fitted with a colour CCD camera. Leica images were captured and analysed using the Leica Imaging Suite software (overlays represent true colours). Micrographs of cells shown in this thesis were captured in 10X, 40X or 63X magnification. All immunofluorescence was validated by testing for non-specific secondary antibody binding.

2.11. Internalisation assay and flow cytometry analysis

Transfected Jurkat cells (refer to section 2.6.1; page 55) (untransfected cells as negative control) were re-suspended in 1 mL of 0.5% BSA/PBS to give cell density of 1 X 10^6 per mL. Cells were washed with 0.5% BSA/PBS twice and re-suspended with 300 µL of 0.5% BSA/PBS. Cells were activated with 15 nM CXCL12 at 37°C for 15 minutes or left untreated as negative control. Cells were then washed with ice-cold 0.5% BSA/PBS twice and were re-suspended in anti-h CXCR4 antibody (R&D systems) (1 in 2000) at 4°C for 1 hour (resuspension with 0.5% BSA/PBS as negative control). Cells were gently washed twice in ice-cold 0.5% BSA/PBS and then incubated at 4°C for 1 hour with secondary antibody goat anti-Mouse IgG (H+L), F(ab')2 fragment, CF 647 (Sigma Aldrich) (1 in 200). Stained cells were gently washed twice with 0.5% BSA/PBS and gated to exclude dead cells. Differences in cellular fluorescence were then determined and quantified using BD FACSCalibur (BD Biosciences) and data analysed using CellQuest software version 3.1 (Becton Dickinson, San Jose, CA).

2.12. Western blotting

2.12.1. Sample preparation

5 X 10^5 cells were spun down at 1800g for 5 minutes and the supernatant was removed. Cells were then either frozen as a pellet for later analysis or used immediately. On the day of analysis, the pellet was defrosted (where necessary) and 40 µL of lysis/ sample buffer (Mammalian Protein Extraction Buffer, GE Healthcare Life Sciences) was added and mixed until a homogenous consistency was reached. Mixture was incubated in ice for at least 30 minutes to promote cell lysis. The lysis mixture was then immediately spun down at 14000g for 10 minutes. The supernatant was removed in preparation for gel electrophoresis and the pellet was discarded. Protein concentration of samples were then measured by the Nanodrop spectrometer system (ThermoFisher Scientific). The lysed sample was placed in boiling water for 5 minutes to denature proteins further before use for SDS-PAGE.

2.12.2. SDS-PAGE sample separation

SDS-PAGE gels consisted of a stacking phase for sample loading (0.1089g Tris, SDS 2% pH 8.8, 4.5% acrylamide v/v, 0.1% ammonium persulphate w/v, 0.01% TEMED v/v) which constituted the top 20% gel and a 12% acrylamide running phase (0.67155g Tris/SDS 2% pH 8.8, 12% acrylamide v/v, 0.1% ammonium persulphate w/v, 0.01% TEMED v/v). 20 µL of sample was loaded into the stacking phase wells with a Hamilton microsyringe along with 5µL Pierce pre-stained protein MW marker (ThermoFisher Scientific) as ladder. Unused wells were loaded with 20µL loading buffer (4% SDS, 0.02% bromophenol blue, 20% glycerol, 80 mM Tris, pH 6.8) to allow identification of the elution front. Gels were run at a constant 30 mA until the elution front reached the running gel when the current is increased to 50 mA constant until the elution front ran off the gel, at which point electrophoresis was complete.

2.12.3. Protein transfer

The gel plates were removed from the clamp assembly and separated so that the gel remained intact on one plate. The gel was then transferred to a nitrocellulose

membrane (Whatman) pre-soaked with transfer buffer. The membrane and gel were placed between sheets of (Whatman) filter paper pre-soaked in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3), sufficient sheets were used to allow a good contact between electrodes of the transfer plate in the semi-dry blotter kit (Sigma-Aldrich). The transfer was undertaken at 15 volts constant for 45 minutes. Successful protein transfer was evaluated by the transfer of the protein marker to the nitrocellulose membrane.

2.12.4. Immunostaining and imaging

After protein transfer was verified the nitrocellulose membranes were initially blocked for non-specific binding with PBS-T (0.5% v/v Tween-20 in PB) containing 5% w/v low-fat milk powder (Marvel) for 1 hour at room temperature. Blocking buffer was then removed and the relevant primary antibody (table 2.4) was diluted in blocking buffer before overnight rolled-agitation at 4°C. The following day the primary antibody was removed, and the membrane washed 3 times for 5 minutes with PBS-T before addition of the relevant secondary peroxidase tagged Ab was added at 1:5000 in 10 mL blocking buffer. After 1-hour roller-agitation, the secondary antibody solution was removed, and the membrane was washed 3 times for 5 minutes in a tray with gentle shaking to facilitate flattening. Proteins were visualised by addition of Pierce ECL Western Blotting Substrate (ThermoFisher Scientific) after which the membrane was sandwiched between 2 acetate sheets and imaged using the G-Box imaging system (Syngene, UK) set to automatically calculated exposure. Images were captured using Gene-snap software (Syngene, UK).

2.12.5. Membrane stripping

Antibodies were removed from the membrane before a second stain by incubation with Millipore Stripping Solution (Millipore, Temecula California) at room temperature for 15 minutes before blocking and re-probing for immunostaining as described previously (section 2.11.4).

2.13. Bioluminescence Resonance Energy Transfer (BRET)

Cells were transfected with plasmids DNA CXCR4-RLuc3 (donor) and h-arrestin3-GFP10 (acceptor) and also maintained as described previously (refer to section 2.6.1; page 55). Transfected cells were then seeded in 96-well plates (with clear bottoms that had been treated with poly-D-lysine) (Corning) and left in culture for 24 hours before being processed for BRET assay. Transfection was assessed by visualising localisation of fluorescent h-arrestin3-GFP10 in cells using Leica DMII fluorescence microscope with GFP filter cube. Luciferase substrate solution, coelenterazine 400a (DeepBlueC) (Biotium), stock was reconstituted in ethanol for BRET assay and EnduRen (Promega) stock was reconstituted in DMSO. For routine BRET measurements, cells were washed with BRET buffer (PBS, 0.5 mM $MgCl_2$, 0.01% BSA) 24 to 48 hours after transfection and luciferase substrate solution, DeepBlueC (Biotium) or EnduRen (Promega) was added to a final concentration of 5 µM or 60 µM respectively in BRET buffer (PBS, 0.5 mM $MgCl_2$, 0.1% glucose). DeepBlueC (Biotium) was added just before detecting emission from that well and EnduRen (Promega) was added to live cells at least 1.5 hours before BRET detection. Readings were then collected using Clariostar Monochromator (BMG Labtech) measuring signals detected in the 410±40 nm and 515±15 nm windows for luciferase and GFP10 light emissions. The BRET signal is determined by calculating the ratio of the light intensity emitted by the h-arrestin3-GFP10 over the light intensity emitted by CXCR4-RLuc3. The values were corrected by subtracting the background BRET signal detected when the CXCR4-RLuc3 construct was expressed alone. To assess the effects of ligands, CXCL12 was added at the 0.01 nM, 0.1 nM, 1 nM, 5 nM and 10 nM and incubated at 37°C for 5 minutes before the addition of luciferase substrate solutions and BRET reading. For acquisition of full BRET spectra, cells were transfected as described above with different amounts of h-arrestin3-GFP10 for a given quantity of CXCR4-RLuc3 (0.1µg). Cells were detached and re-suspended in BRET buffer (PBS, 0.5 mM $MgCl_2$, 0.01% BSA). 200,000 cells were seeded in 100 µL of BRET buffer (PBS, 0.5 mM $MgCl_2$, 0.01% BSA) in a 96-well plate with a clear bottom (Corning), and BRET scan was performed in FLUOstar Optima (BMG Labtech) by reading luminescence between 480 – 520 nm after addition of DeepBlueC for cells expressing [acceptor]/[donor] ratios.

2.14. Curve prediction and statistical analysis

All data were analysed using GraphPad Prism 6 and represent at least 3 independent experiments (unless stated otherwise). All concentration response sigmoidal curves used to calculate EC_{50} and IC_{50} values were fitted assuming a Hill coefficient of 1. Statistical significance was determined using unpaired student t-tests where applicable or one-way ANOVA analysis of variance followed by Bonferroni or Dunnett's multiple comparison as post-tests where 3 or more data sets were to be analysed. A significant value of 95% was used with p values indicated as follows $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***. P values above 0.05 were therefore considered as not significant (ns). All error bars represent standard error of the mean value (S.E.M).

CHAPTER 3: Characterisation of CXCR4 activation

3.1. Introduction

Chemokines are chemotactic cytokines, a superfamily of small secreted cytokines that were initially characterised through their ability to prompt the migration of leukocytes. Chemokine receptors and their ligands were shown to be interesting therapeutic targets due to their involvement in various immune-related diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, chronic obstructive pulmonary disease, HIV-1 infection and cancer [1,270]. Attention has been focused on the chemokine receptors expressed on cancer cells as distinct patterns of chemokine/receptor expression are linked to many diseases, including the progression of cancer and cancer cell migration which show similarities to leukocyte trafficking [9]. Emerging evidence has shown that chemokines are involved in cancer progression through specific chemokine/chemokine receptor pairing in tumour metastasis and the shaping of tumour microenvironment in favour of tumour growth and metastasis [3,11]. Involvement of chemokines in chemokine receptor signalling also contributes to the survival and proliferation of cells, which is important for cancer cells to adapt to foreign environments [11]. The chemokine receptor CXCR4 is most commonly found on human and murine cancer cells, and it is primarily activated by the chemokine CXCL12 [2,11]. This ligand can induce intracellular signalling through several divergent pathways initiating signals related to chemotaxis, cell survival, and/or proliferation, increase in intracellular calcium and gene transcription. Interactions between CXCR4 and CXCL12 have a crucial role in the migration and patterning of many embryonic cell lineages, the mobilisation of HSCs and the migration of naïve lymphocytes [1]. The expression of CXCR4 on malignant epithelial and hematopoietic cells suggests that CXCR4/CXCL12 pathway may influence the biology of cancer and play an important role in directing the metastasis of $CXCR4^+$ tumour cells to organs that express CXCL12 [2]. It is essential to study the network of chemokines and their receptors as they influence the development of primary tumours and metastases but due to its complexity, it is still not yet fully understood.

3.2. Chapter aims

Hypothesis: We hypothesised that different chemokines illicit different signalling pathway responses in different cell types.

Aims: The aim of this chapter is to characterise CXCR4 activation using different chemokine ligands and to identify a 'route' by which activated CXCR4 receptors transduce signals to chemotaxis. Most chemokines bind to multiple receptors and the same receptor may bind to more than one chemokine, suggesting further studies of CXCR4 and its activation with variety, including chemically modified chemokines would be interesting. There is accumulating evidence that chemokines may display their own pattern of activation of signalling pathways, suggesting that different chemokines can play different roles [270]. Ultimately, this would aid in mapping the chemokine network and the signalling events upon chemokine ligand binding on CXCR4 activation. This will allow relation of observations to specific signalling axis and allow comparison to the relevant literature. By understanding the involvement of chemokines and their receptors in migration of cells, novel therapeutic targets to stop cancer metastasis may be discovered.

3.3. Results

3.3.1. Expression of chemokine receptors in MCF-7 cells

MCF-7 cells are adherent, non-invasive breast carcinoma cells, and they have been shown to express chemokine receptors, such as CXCR4, by quantitative RT-PCR analyses [4,192]. High levels of CXCR4 expression in human breast cancer correlates with metastasis to tissues, such as lymph nodes, bone marrow, liver and lung, that secrete CXCL12 [4,8]. CXCL12 is a homeostatic chemokine, and the major function is to regulate hematopoietic cell trafficking and secondary lymphoid tissue architecture [2].

MCF-7 cells were harvested and washed with calcium flux buffer (refer to section 2.8; page 57). MCF-7 cells were incubated with Fura-2 AM, which can pass through the cell membrane and fluoresce upon binding to calcium. This makes it a useful tool in the quantification of calcium signalling occurring upon receptor activation. CXCL12 was demonstrated to induce calcium responses from MCF-7 cells in a dose-dependent manner, with an EC_{50} of 93.61 nM (assuming Hill coefficient of 1) (figure 3.1).

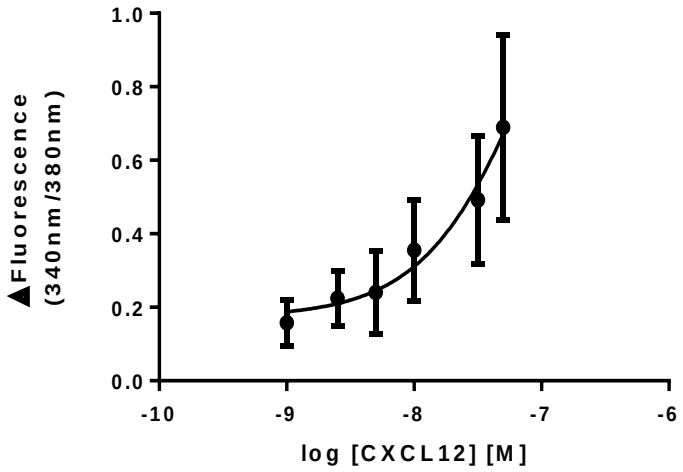


Figure 3.1: Concentration response curve for MCF-7 cells upon CXCL12 activation. Data is expressed as changes in fluorescence ratio (340nm/380nm) where the basal fluorescence, prior to the addition of CXCL12, is subtracted from peak fluorescence following addition of CXCL12. Data represent mean ± SEM from at least three independent experiments.

To confirm that MCF-7 cells are suitable to study the CXCR4/CXCL12 signalling axis, we did preliminary experiments to confirm cell surface expression of different chemokine receptors on MCF-7 cells by activating them with a variety of chemokines. In this study, we used CCL3, CXCL8 and CXCL12.

CCL3, also known as MIP-1α, is a cytokine belonging to CC chemokine family with inflammatory and chemokinetic properties [271]. They are known to be involved in the acute inflammatory state in the recruitment and activation of leukocytes [272,273]. CCL3 binds to the receptors CCR1 and CCR5 [271]. CCL3 plays an important role in the pathogenesis of various inflammatory diseases and conditions that exhibit bone resorption, resulting in good diagnostic potential for the detection of several inflammatory diseases such as rheumatoid arthritis [273]. CCL3 is one of the chemokines that is most widely studied because it has been shown that it is one of the major HIV-suppressive factors produced by CD8+ T-cells [271,274].

CXCL8, also known as IL-8, is a chemokine that attracts neutrophils, basophils, and T-cells but not monocytes [275]. It has been shown to be involved in neutrophil activation [276]. CXCL8 is released from several cell types in response to an inflammatory stimulus, including epithelial cells, airway smooth muscle cells and endothelial cells [276–279]. CXCL8 initially is produced as a precursor peptide of 99 amino acids which then undergoes cleavage to create several active CXCL8 isoforms [275]. CXCL8 receptors that could contribute to CXCL8-mediated tumorigenic and angiogenic responses include CXCR1 and CXCR2 [275], and since there are different CXCL8 isoforms, it has been shown that CXCL8 (7-77) has a higher affinity for receptors CXCR1 and CXCR2 as compared to CXCL8 (1-77) [278].

CXCL12, also known as SDF-1, is produced in two forms; CXCL12a (SDF-1α) and CXCL12b (SDF-1β), by alternative splicing of the SDF-1 gene [280,281]. In our laboratory, the isoform that we usually use in our assays is SDF-1α. CXCL12 is a chemoattractant, and it activates CXCR4 to induce a rapid and transient rise in the level of intracellular calcium and chemotaxis [282,283].

Figure 3.2 demonstrates that CCL3, CXCL8 and CXCL12 are all capable of inducing a release of intracellular calcium response upon activation of their chemokine receptors

in MCF-7 cells. The literature suggests that these signalling pathways could occur through receptors CXCR1 and CXCR2 upon CXCL8 activation; CCR1 and CCR5 upon CCL3 activation; and CXCR4 upon CXCL12 activation. Mueller *et al.* (2001) reported that MCF-7 cells express upregulated levels of CCR5, CXCR2 and CXCR4 through flow cytometric analyses and quantitative RT-PCR analyses [4]. This explains the intracellular calcium signalling observed upon activation of chemokine receptors in MCF-7 cells in figure 3.1. This data shows that we can perform specific chemokine receptor studies using these different chemokines in MCF-7 cells to study downstream signalling cascades, which may result in chemokine-induced migration of these cell types.

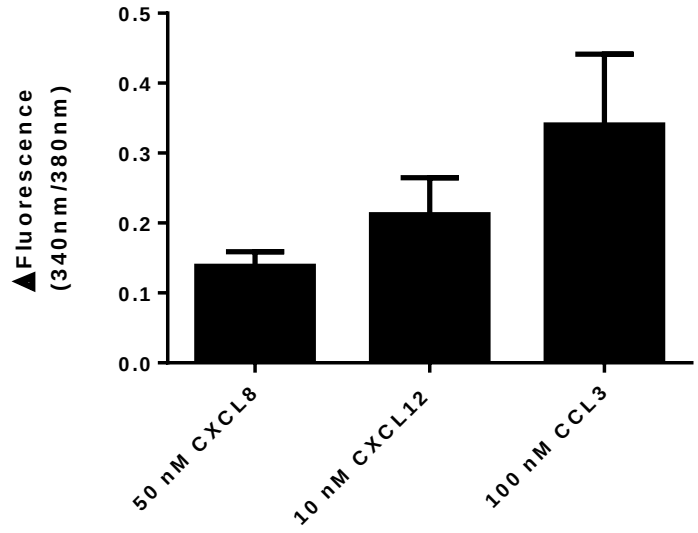


Figure 3.2: Comparison of intracellular calcium response upon activation of different chemokine receptors by different chemokines in their working concentrations. Data is expressed as changes in fluorescence ratio (340nm/380nm) where the basal, before addition of chemokine, is subtracted from peak fluorescence after addition of chemokine. Data represent mean ± SEM from at least three independent experiments.

3.3.2. CXCL12 N-His Tag binding to CXCR4 promotes intracellular calcium response in MCF-7 cells but not chemotaxis in suspension leukemic T-lymphocytes, Jurkat cells

As discussed previously, Mueller *et al.* (2001) confirmed expression of CXCR4 was markedly upregulated and that there is strong cell-surface expression of CXCR4 on MCF-7 cells [4]. CXCR4 is a receptor for CXCL12 that transduces a signal by increasing intracellular calcium ion levels and enhancing MAPK1/MAPK3 activation and finally chemotaxis [257,284]. The binding of CXCL12 to CXCR4 induces intracellular signalling through several divergent pathways initiating signals related to chemotaxis, cell survival and/or proliferation, increase in intracellular calcium and gene expression [2,280,285].

Chemokines are characterised by the presence of four conserved cysteines, which form two disulphide bonds [11]. The CXCL12 proteins belong to a group of CXC chemokines, whose initial pair of cysteines are separated by one intervening amino acid [5]. CXCL12 is strongly chemotactic for lymphocytes and has been implicated as an important cell co-ordinator during development [82,283]. In a collaboration with Professor K. Schmitz (TU Darmstadt), we were given some CXCL12 N-His Tag and wanted to investigate if it can induce intracellular signalling via CXCR4. The recombinant human CXCL12 consists of 83 amino acids and predicts a molecular mass of 9.83 kDa (figure 3.3).

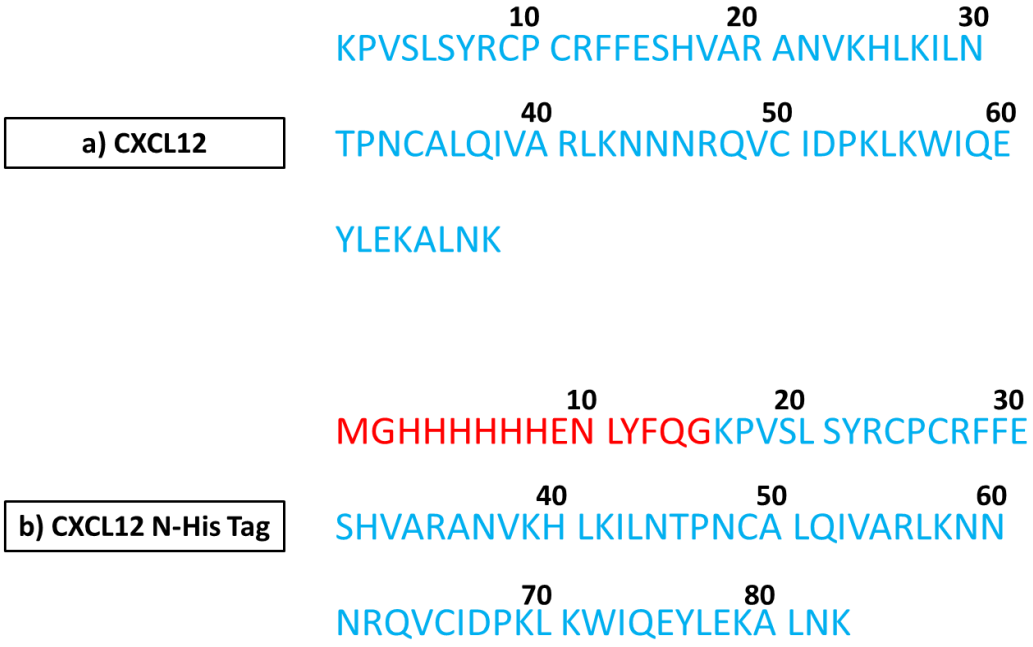


Figure 3.3: Amino acid sequence of (a) CXCL12, containing 68 amino acid residues (marked blue) and (b) CXCL12 N-His Tag, containing 83 amino acid residues. Difference between the two recombinant human SDF-1α is the 15 amino acids His-Tag (marked red) that was fused at the N-terminus in CXCL12 N-His Tag, which makes this protein have a molecular mass of 9.83 kDa compared to the molecular mass of CXCL12 of 8.0 kDa.

MCF-7 cells were incubated with Fura-2 AM at 37°C and intracellular calcium response stimulated by different concentrations of CXCL12 N-His Tag vs 10 nM CXCL12 positive control was measured after 60 minutes of incubation. Binding of CXCL12 N-His Tag to CXCR4 in MCF-7 cells was demonstrated to promote intracellular calcium signalling. CXCL12 N-His Tag EC_{50} for calcium release was calculated as 274 nM (assuming Hill coefficient of 1) (figure 3.4a). Calcium signalling increases as the concentration of CXCL12 N-His Tag increases (figure 3.4a). MCF-7 cells were treated with 10 nM CXCL12 as positive control to show that cells were able to signal. Calcium flux was quantified and this increase in intracellular calcium suggested that there was chemokine activity upon binding. It is hypothesised that this is achieved via the Gβγ subunit. This then triggers PLC activation and formation of IP3 and DAG, which results in mobilisation of calcium from intracellular stores [70]. Figure 3.4b confirmed that there was calcium

signalling in MCF-7 cells upon CXCR4 activation by varying concentration of CXCL12 N-His Tag when compared to 10 nM CXCL12 positive control.

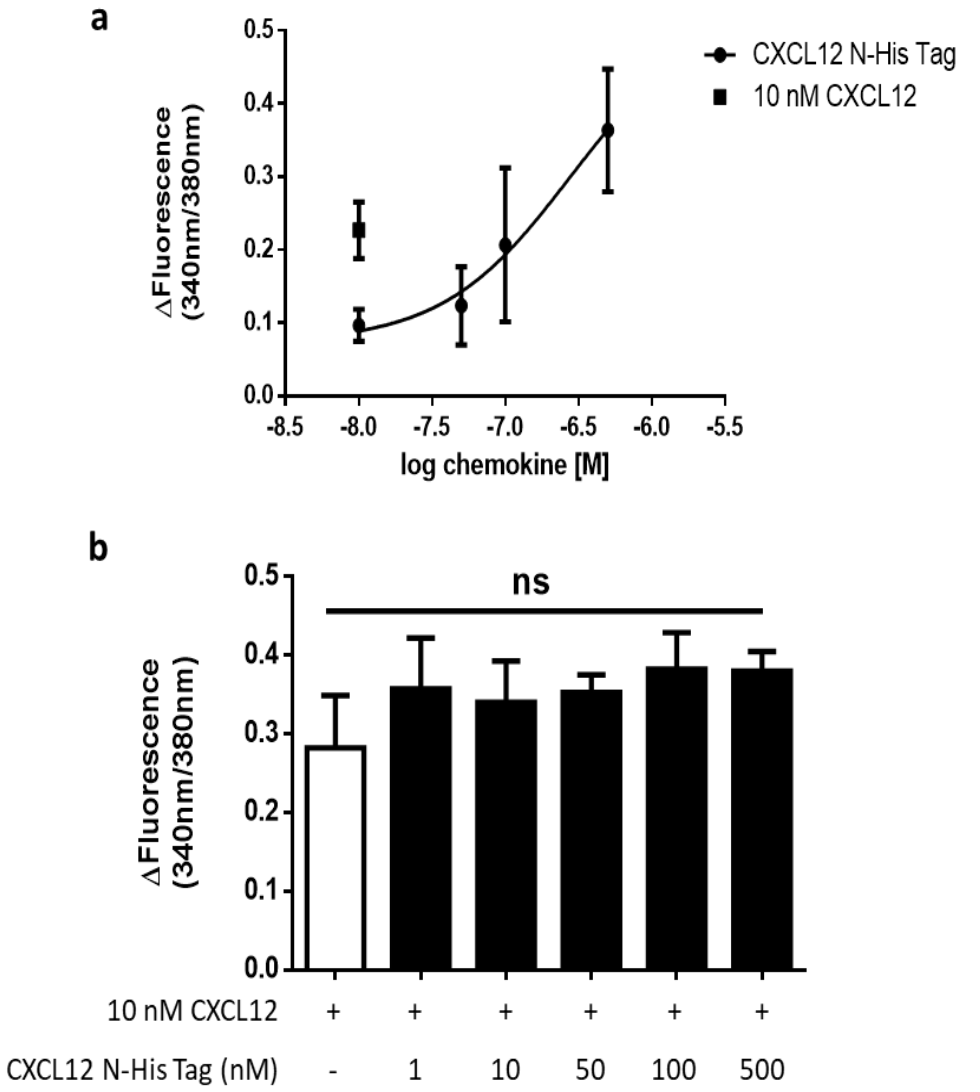


Figure 3.4: CXCL12 N-His Tag binds to CXCR4 to induce intracellular calcium response. (a) Concentration response curve for MCF-7 cells upon CXCL12 N-His Tag activation. (b) Calcium flux response: MCF-7 cells upon CXCL12 activation in presence of different concentrations of CXCL12 N-His Tag as antagonist vs positive control 10 nM CXCL12. Data is expressed as changes in fluorescence ratio (340nm/380nm) where the basal fluorescence prior to the addition of CXCL12 is subtracted from peak fluorescence following addition of CXCL12. Data represent mean ± SEM from at least three independent experiments (One-way ANOVA, Bonferroni multiple comparison, ns = not significant, $p>0.05$).

Since our group has previously shown that chemotaxis can occur independently of intracellular calcium release [142] and that there is differential regulation of signalling

in different cell lines [286], we wanted to investigate if the binding of CXCL12 N-His Tag and CXCR4 could also induce migration in Jurkat cells using chemotaxis assays.

Jurkat cell line is an immortalised T lymphocyte cell line that was originally obtained from the peripheral blood of a boy with T cell leukaemia [287]. One of the reasons why Jurkat cell line has been widely used is due to the expression of various chemokine receptors, susceptible to viral entry, particularly HIV, where it was identified that HIV receptor (CD4) and the co-receptors (CXCR4 and CCR5) HIV binding to susceptible target cells [257,271].

Jurkat cells were treated with different concentrations of CXCL12 N-His Tag and CXCL12 as positive control, and chemotaxis assays were analysed after 5 hours of incubation. Figure 3.5a shows that CXCL12 N-His Tag has no chemotactic activity on Jurkat cells compared to the positive control of 1 nM CXCL12, even at high concentrations.

Since CXCL12 N-His Tag showed that they may have binding activity on CXCR4 as they induced intracellular calcium signalling (figure 3.4a), we would like to investigate if they had an effect on CXCR4/CXCL12 signalling axis. Even though CXCL12 N-His Tag showed no chemotactic activity on Jurkat cells, it would be interesting to see if they have a role in binding of chemokine CXCL12 to chemokine receptor CXCR4. At increasing concentrations, CXCL12 N-His Tag seems to act as an antagonist that blocks CXCR4-mediated chemotaxis induced by the endogenous ligand, CXCL12 (figure 3.5b).

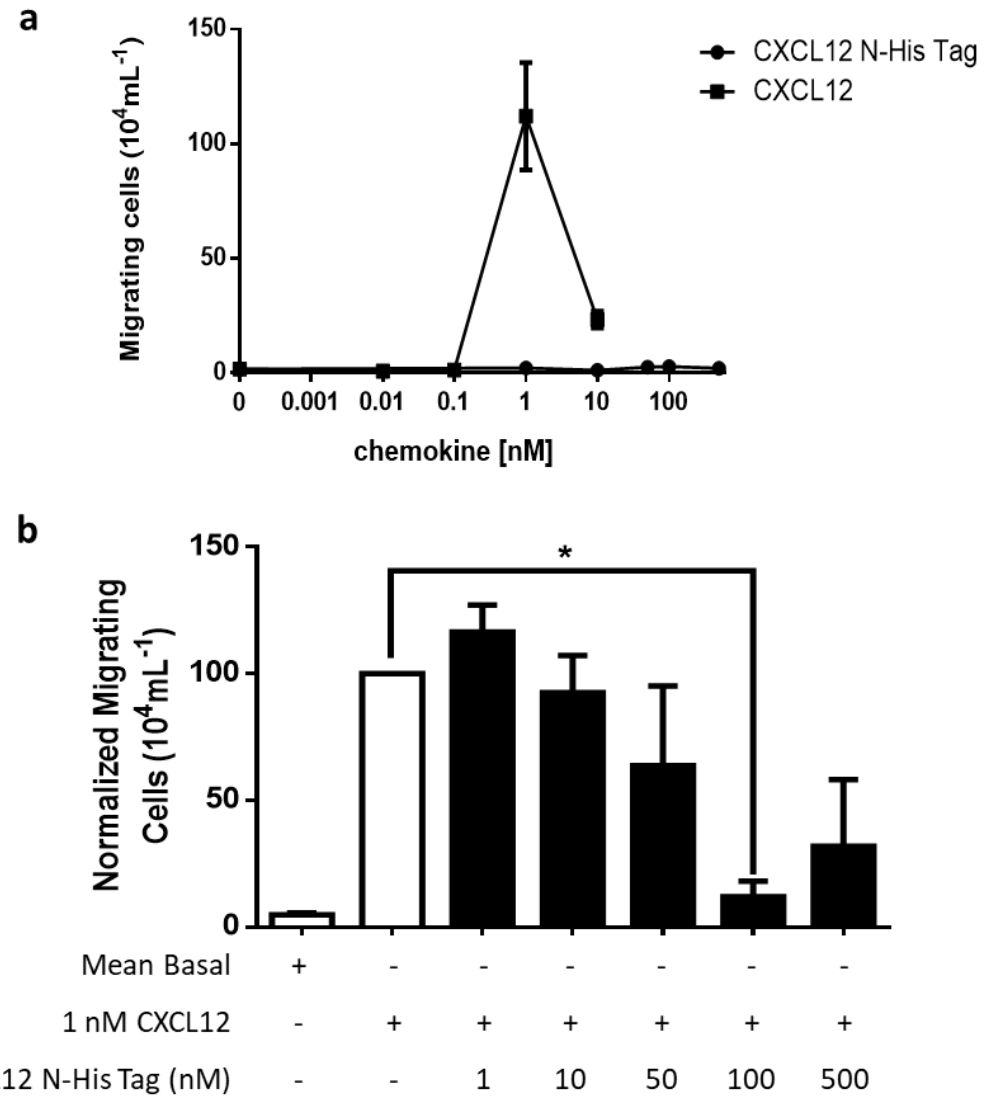


Figure 3.5: CXCL12 N-His Tag does not induce CXCR4-mediated chemotaxis and it acts as an inhibitor in CXCL12-induced chemotaxis in Jurkat cells. (a) Concentration response plot of Jurkat migration towards 0, 0.01, 0.1, 10, 50, 100 and 500 nM CXCL12 N-His Tag vs positive control 0, 0.01, 0.1, 1 and 10 nM CXCL12. (b) Migration of Jurkat cells was induced in the presence or absence of CXCL12 N-His Tag, which was made to concentrations stated in mixture with 1 nM CXCL12. Data represent mean ± SEM from at least three independent experiments (One-way ANOVA, Bonferroni multiple comparison, * = $p \leq 0.05$).

Figure 3.5a shows that the optimal dose of CXCL12 is 1 nM. There was reduced chemotactic activity when Jurkat cells were treated with more than 1 nM CXCL12 in a dose-dependent manner (figure 3.5a).

Figure 3.5b shows that when cells were incubated with both 1 nM CXCL12 and increasing concentrations of CXCL12 N-His Tag, there was a trend towards inhibition

of cell migration in a dose dependent manner, which was significant at 100 nM CXCL12 N-His Tag.

3.3.3. Characterisation of chemokine-mediated chemotaxis in Jurkat cells

Here, we have chosen the Jurkat cell line to characterise chemotaxis signalling in response to different chemokines. As discussed previously, Jurkat cells have been widely used in chemokine research as they express various chemokine receptor.

Chemokines that were used in our lab to perform chemotaxis assays in Jurkat cells were CCL1, CCL2, CCL3, CCL5, CCL8, CCL23, CXCL11 and CXCL12. These are summarized in table 3.1.

Table 3.1: Chemokines

Chemokines	Description	Receptors	Refs
CCL1 (I-309)	• chemotactic cytokine for monocytes, NK cells and immature B cells and dendritic cells but not neutrophils • a small glycoprotein secreted by activated T cells	CCR8	[288–290]
CCL2 (MCP1)	• recruits monocytes, memory T cells and dendritic cells, but not neutrophils or eosinophils to the sites of inflammation produced by either tissue injury or infection • ability to bind to receptors on the surface of leukocytes targeted for activation and migration	CCR2	[286–288]
CCL3 (MIP-1α)	• useful as a biomarker as it performs various biological functions such as recruiting inflammatory cells, wound healing, inhibition of stem cells, and maintaining effector immune response	CCR1, CCR5	[273]
CCL5 (RANTES)	• chemokine for T cells, eosinophils and basophils, and plays an active role in recruiting leukocytes into inflammatory sites • has been demonstrated to have a critical role in T-lymphocyte activation and as one of the major HIV-suppressive factors produced by CD8+ T cells	CCR1, CCR3, CCR5	[294–297]
CCL8 (MCP2)	• uses multiple cellular receptors to attract and activate human leukocytes • has been shown to act as a potent inhibitor of HIV-1 due to its high-affinity binding to the receptor CCR5, which is known as one of the major co-receptors for HIV	CCR2, CCR5	[1,295, 298]
CCL23 (MIP-3)	• highly chemotactic for resting T cells and monocytes, and slightly chemotactic for neutrophils, but not for activated lymphocytes	CCR1	[295]
CXCL11 (IP-9)	• chemotactic for interleukin-activated T-cells • has been shown to exhibit effects on its target cells by interacting with CXCR3, with a higher affinity than CXCL9 and CXCL10	CXCR3	[300,301]
CXCL12 (SDF1-α)	• has been chemokine of interest as CXCR4/CXCL12 signalling axis is involved in numerous cancers. CXCL12 plays a key role in several aspects of tumour progression including angiogenesis, metastasis and survival.	CXCR4, ACKR3	[55,302–305]

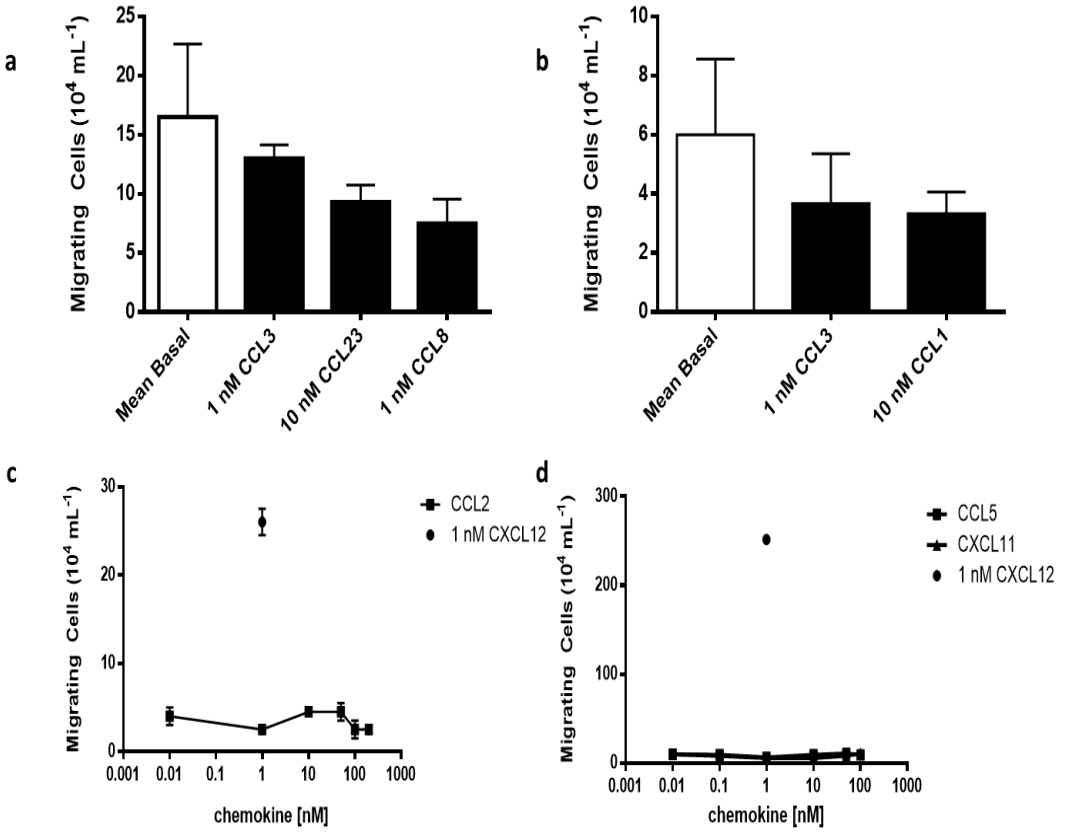


Figure 3.6: Comparison of migration of Jurkat cells in different chemokines in chemotaxis assays after 5 hours of incubation. (a) Migration of Jurkat cells measured in the presence or absence 1 nM CCL3, 10 nM CCL23 and 1 nM CCL8 (b) Migration of cells measured in the presence or absence 1 nM CCL3, 10 nM CCL1 and 10 nM CXCL12 (c) Concentration response plot comparing migration of cells observed towards 0.01, 1, 10, 50, 100, 150 nM CCL2 to positive control 1 nM CXCL12. Note: positive control stimulated migration is 10-fold lower than expected (d) Concentration response plot of Jurkat comparing migration towards 0.01, 0.1, 1, 10, 50, 100 nM CCL5 and CXCL11 to positive control 1 nM CXCL12. Data represent mean ± SEM from at least three independent experiments. Note: Experiments were performed by visiting students in our laboratory.

Jurkat cells were treated with different chemokines at their working concentrations in chemotaxis assays and analysed after 5 hours incubation (refer to section 2.9.1; page 58). Figure 3.6 shows that Jurkat cells did not migrate towards CCL1, CCL2, CCL3, CCL5, CCL8, CCL23 or CXCL11. There may be several explanations for these observations. The first explanation is that Jurkat cells may not express these chemokine receptors on their cell surface. Secondly, it may be that these interactions do not induce chemotactic activity in this cell type. GPCRs are key mediators of intracellular signalling, initiating signals elating to chemotaxis, cell survival and/or proliferation, increase in intracellular calcium and gene transcription, but these are cell-type,

receptor-type and ligand-type dependent interactions [27,306]. Hence, there might be activation of receptors by these ligands in the cells, but it doesn't induce intracellular signalling to chemotaxis. To confirm if this is the case, further experiments of immunofluorescence staining of primary antibodies should to be done to ensure the expression of the receptors that interact with the chemokines, and that the effects of the signalling are due to activation of the receptors by the chemokines.

The third explanation to this observation may be due to the use of inappropriate concentration of chemokines. Chemokine induced cell migration usually generates a bell-shaped chemotactic dose-response curve [307]. There might be a possibility that the concentration of chemokines used were not within the range to induce chemotactic signalling. Increasing concentration of chemokine could have been done but that would have been too expensive to continue.

These results have shown that the CXCR4/CXCL12 signalling axis can be studied using Jurkat cell lines, and that Jurkat cell lines are not suitable to study downstream migratory signalling cascades of other chemokine receptors.

3.3.4. CXCL14 acts as an inhibitor of CXCL12-induced chemotaxis in Jurkat cells

CXCL14, also known as breast and kidney-expressed chemokine (BRAK), is a homeostatic chemokine with unknown chemokine receptor selectivity and is expressed in epithelial cells in normal tissue in the absence of inflammatory stimuli [308]. Its expression is usually downregulated in inflammatory setting, which suggests that CXCL14 plays an important role in the maintenance of tissue homeostasis and that it is a highly selective monocyte chemoattractant [309].

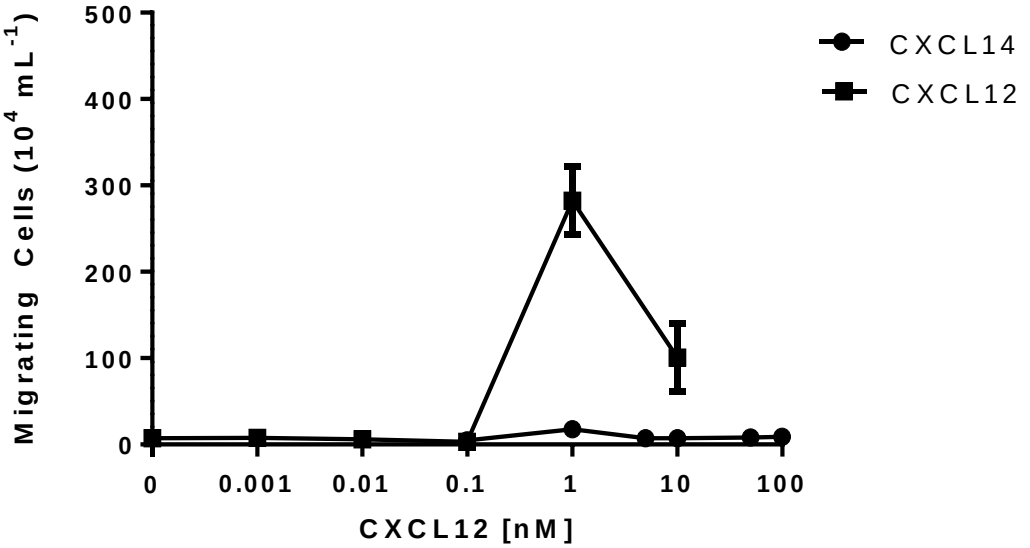


Figure 3.7: CXCL14 does not induce migration of Jurkat cells. Concentration response plot of Jurkat cells migrating towards 0, 0.001, 0.01, 0.1, 1, 5, 10, 50, 100 nM CXCL14 vs 0, 0.001, 0.01, 0.1, 1, 10 nM CXCL12 as positive control. Data represent mean ± SEM from at least three independent experiments.

There was no substantial migration of Jurkat cells towards increasing concentrations of CXCL14 compared to increasing concentrations of CXCL12 which acted as positive control (figure 3.6). This can be due to CXCL14 not binding and activating CXCR4, but in recent years, studies have shown that CXCL14 specifically binds to CXCR4 with high affinity [310,311], therefore, this result cannot be explained by that hypothesis. Alternative hypothesis of the data suggests that CXCL14 is not a chemotactic cytokine for this cell type as intracellular signalling events can be cell-type specific [286,312,313]. Despite the potential clinical significance of CXCL14, the molecular mechanisms behind the physiological functions of CXCL14 are poorly understood.

Collins *et al.* (2017) have shown that epithelial chemokine CXCL14 synergises with CXCL12 via allosteric modulation of CXCR4 [308]. Since we have shown that there is differential regulation of cell migration in different chemokine receptors in different cell types [286,314] in our previous studies, it is useful to investigate the role of CXCL14 in CXCL12-induced migration as CXCL14 binds specifically to CXCR4 as discussed previously [310,311].

In studying the relationship between CXCL14 and CXCL12, we tried to establish if there was crosstalk between them in the CXCR4-mediated downstream signalling response.

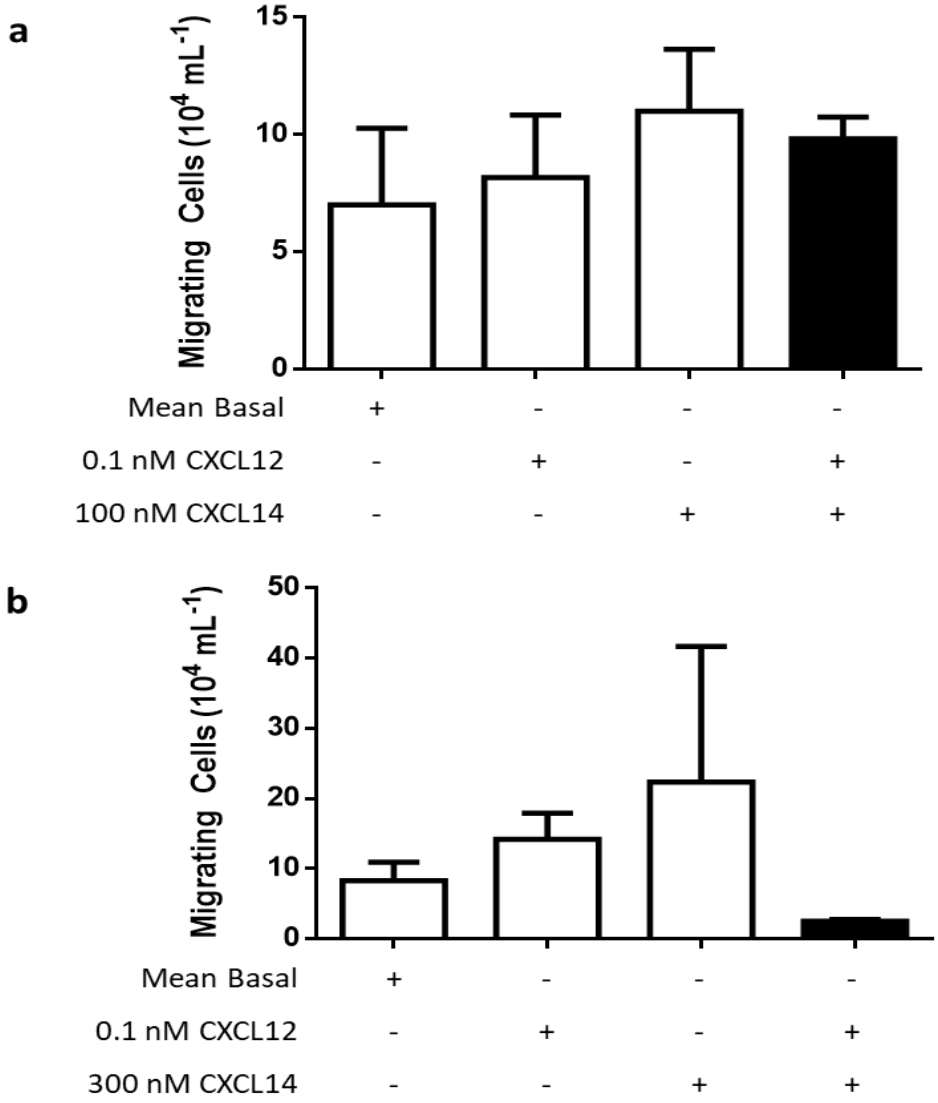


Figure 3.8: CXCL14 does not induce migration and doesn't have a synergistic effect with CXCL12 in CXCR4-mediated migration of Jurkat cells. (a) Migration of Jurkat cells was induced in the presence or absence of 0.1 nM CXCL12, 100 nM CXCL14 and combination mixture of 0.1 nM CXCL12 and 100 nM CXCL14. (b) Migration of Jurkat cells was induced in the presence or absence of 0.1 nM CXCL12, 300 nM CXCL14 and combination mixture of 0.1 nM CXCL12 and 300 nM CXCL14. Data represent mean ± SEM from at least three independent experiments.

Here, we have lowered the concentration of CXCR4 endogenous ligand, CXCL12 and carried out chemotaxis assays of Jurkat cells with 0.1 nM CXCL12 instead of working concentration, 1 nM CXCL12. Since there was evidence of synergy between CXCL12 and CXCL14 [308], we wanted to investigate the relationship between CXCL14 and CXCL12 with different combinations of chemokine concentrations. This would determine if

CXCL14 would co-operate with CXCL12 to produce an additive effect in the induction of cell migration.

Figure 3.8 shows that Jurkat cells do not migrate towards 0.1 nM CXCL12, 100 nM CXCL14 or 300 nM CXCL14 independently, or in assays induced with combination mixture of 0.1 nM CXCL12 and 100 nM CXCL14, or 0.1 nM CXCL12 and 300 nM CXCL14 (figure 3.8). We expected to observe no migration towards 0.1 nM CXCL12 and also with both 100 nM and 300 nM CXCL14, as demonstrated previously in figure 3.7. However, in contrast to a previous study [308], we observed no migration of cells in either combination mixtures of 0.1 nM CXCL12 and 100 nM CXCL14; or 0.1 nM CXCL12 and 300 nM CXCL14. Currently, this data suggests the lack of synergistic effect between CXCL14 and CXCL12, or the concentrations of CXCL12 and/or CXCL14 were not enough.

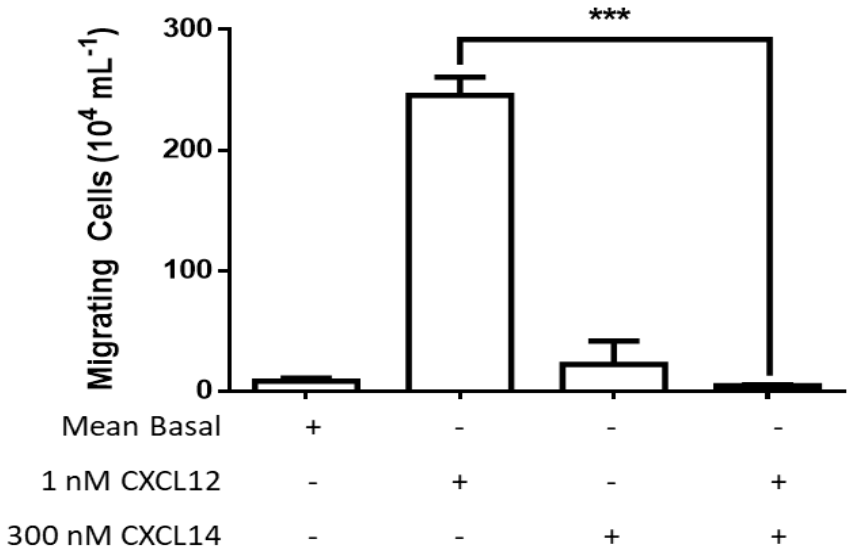


Figure 3.9: CXCL14 does not induce migration and had inhibitory effect towards CXCL12-induced migration of Jurkat cells. Migration of Jurkat cells was induced in the presence or absence of 1 nM CXCL12, 300 nM CXCL12 and combination mixtures of 1 nM CXCL12 & 300 nM CXCL12 in chemotaxis assays after 5 hours of incubation. Data represent mean ± SEM from at least three independent experiments. (One-way ANOVA, Bonferroni multiple comparison, *** = $p \leq 0.01$).

The concentration of CXCL12 was then increased from 0.1 nM to 1 nM to observe any additive effect of cell migration due to the synergistic relationship between CXCL14 and CXCL12. Instead of synergy effects observed as demonstrated in previous study [308], we observed significant inhibition of CXCL12-induced chemotaxis of Jurkat cells when treated with 300 nM CXCL14 compared to the positive control of 1 nM CXCL12.

Analysis of these data suggests that CXCL14 acts as an antagonist to CXCL12 for the chemokine receptor CXCR4 in Jurkat cells.

3.4. Discussion

Chemokines are small proteins that function as chemotactic cytokines by directing cellular migration, and therefore have been shown to have a role in helping leukocytes and other migratory cells to find their way in the body [37]. Chemokines have been widely studied due to their involvement in physiology and homeostasis as well as in the pathogenesis of tumours and their metastasis [12]. Studying the roles of chemokines and their chemokine receptors shows promising potential as appropriate targeted therapy in numerous inflammatory diseases and cancers [4,303–305,315]. Plerixafor (AMD3100) is an example drug that has been approved for clinical treatment, which directly blocks the CXCR4 receptor, showing that it is a very efficient inducer of hematopoietic stem cell mobilisation in both animal and human studies [316–318]. It has also been shown to be associated with Warts, Hypogammaglobulinemia, Immunodeficiency and Myelokathexis (WHIM) syndrome [319,320]. WHIM syndrome is caused by mutations in CXCR4 that result in increased agonist-dependent signalling. McDermott *et al.* (2011) have shown that plerixafor increased absolute lymphocyte, monocyte and neutrophil counts in blood to normal and it can correct panleukopenia, which is an acute usually fatal epizootic disease especially of cats that is caused by parvovirus that causes destruction of white blood cells [320]. Their studies showed that that panleukopenia in WHIM syndrome is caused by CXCR4/CXCL12 signalling-dependent leukocyte sequestration [320].

Infiltrating leukocytes are not the only cells that respond to chemokine gradients in cancers; cancer cells can express chemokine receptors and respond to chemokine gradients [4,10]. Organ-specific metastasis might be governed by interactions between chemokine receptors on cancer cells with metastatic potential and chemokine gradients in target organs, showing that chemokines play a significant role in organ-selective cancer metastasis (figure 3.10) [9,28,86].

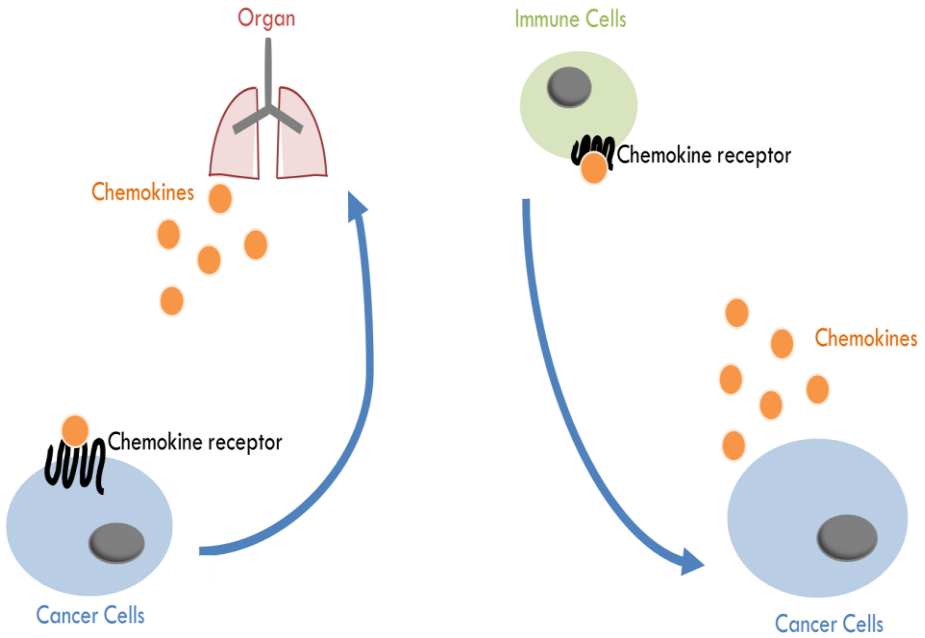


Figure 3.10 (adapted from Koizumi et al. [9]) : Schematic illustrate showing cancer cell migration and metastasis share many similarities with leukocyte trafficking. (a) Chemokine receptors expressed on cancer cells in cancer metastasis, causing it to migrate to organs releasing specific chemokines. (b) Chemokines derived from cancer cells in cancer progression, creating an ideal microenvironment for tumour growth [9].

Many cancer types have upregulated expression of chemokines and chemokine receptors leading to vast chemokine receptor signalling and expression [12]. However, the chemokine receptor that is most commonly found on human and murine cancer cells is CXCR4, and CXCL12 is the only host chemokine ligand for CXCR4 [1,9]. CXCR4 expression is low on normal breast, ovarian and prostate epithelia [4,5,13], and therefore, implies that expression of CXCR4 is a characteristic of malignant epithelial cells and on cells from several hematopoietic malignancies [2]. This suggests that the CXCR4/CXCL12 pathway may influence the biology of cancer and plays an important role in directing metastasis of $CXCR4^{+}$ tumour cells to organs that express CXCL12, including lymph nodes, lungs, liver or bones [9,28].

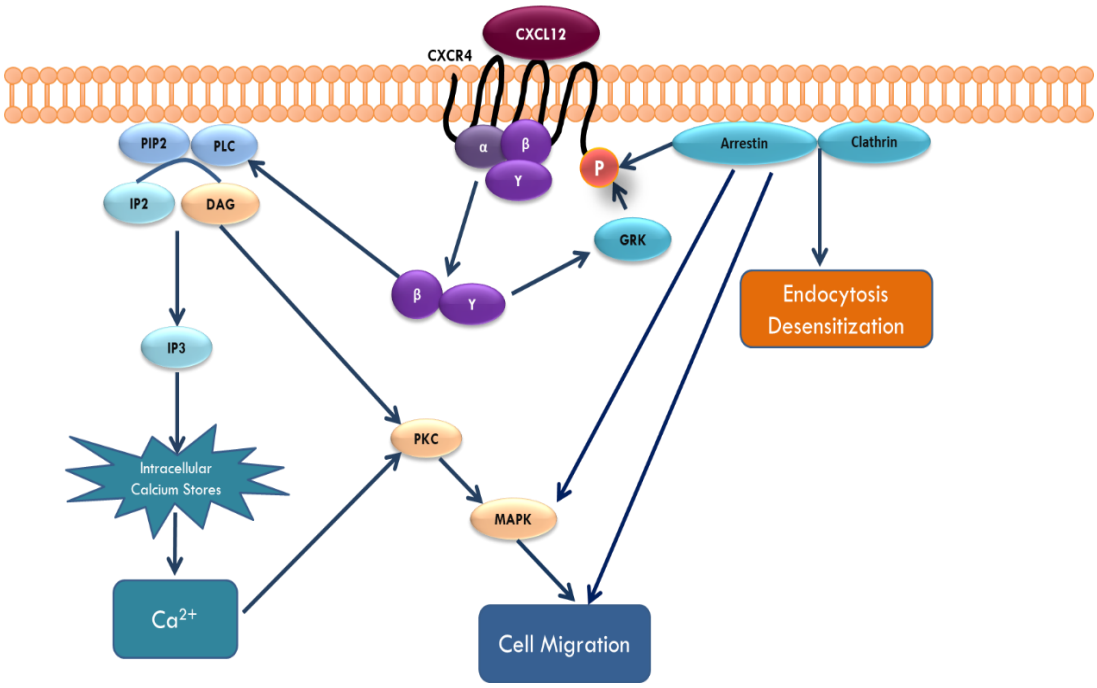


Figure 3.11: Simple overview of chemokine receptor signalling. Calcium release and PKC activation downstream of PLC play an important role in mediating migration [321]. GRK phosphorylation of the C-terminus of chemokine receptors allows arrestins to bind, leading to receptor desensitisation and internalisation. However, arrestin binding also leads to the activation of several proteins including MAPK [11].

As mentioned previously, the binding of CXCL12 to CXCR4 initiates different downstream signalling cascades (figure 3.11), which can result in biological responses such as chemotaxis, cell survival and/or proliferation, increase in intracellular calcium and gene transcription [2]. Activation of GPCRs triggers production of second messenger molecules and activating downstream signalling cascades. Once GPCRs are stimulated, PLC are activated and cleaves PIP2 into DAG and IP3. DAG remains membrane bound and IP3 is released into the cytosol where it binds to specific IP3 calcium channel receptors in the endoplasmic reticulum (ER) [322].

Since the nature of these pathways may be tissue-dependent, triggers of signalling pathways may also differ in cell types [2,286,313]. There was intracellular calcium release upon CCL3, CXCL8 and CXCL12 binding in MCF-7 cells (figure 3.2), suggesting the expression of CCR5, CXCR2 and CXCR4 receptors on breast cancer cells, MCF-7. Since chemotaxis can occur independently of calcium signalling [18,142], investigating chemokine-mediated migration in different cell lines is useful and imperative.

CXCL12 N-His Tag showed an EC_{50} value that was three times higher than CXCL12 (274 nM vs 93.61 nM respectively) (figures 3.1a and 3.4a). CXCL12 N-His Tag exhibited intracellular calcium signalling (figure 3.4a) but no chemotactic activity in Jurkat cells (figure 3.5a), suggesting that there was binding of CXCL12 N-His Tag to the receptor CXCR4 which induces signalling but not chemotaxis. Figure 3.5a also shows that there was reduced chemotactic activity when Jurkat cells were treated with more than 1 nM CXCL12. This may be due to loss of directional cues for the cells to migrate towards as discussed in introduction (refer to section 1.7; page 42). As difference in chemokine gradient no longer exists at high chemokine concentrations, cells would stop moving because there is a loss of chemotactic guidance.

These observations may also be due to the dimerization of CXCL12. A migrating cell will move towards a higher concentration gradient, and at higher concentrations, CXCL12 starts to dimerize, and dimers elicit different signals from the monomers [18,323]. At lower concentrations, CXCL12 monomers are able to promote arrestin recruitment and elicit arrestin-dependent signals such as actin assembly and chemotaxis, but at higher concentrations, CXCL12 starts to dimerize, leading to chemotactic signals being dampened [18].

Previous studies [142,286] have shown that intracellular signalling can occur independently of each other as downstream effectors could be activated differently as intracellular signalling could occur via G-protein-dependent or arrestin-dependent signalling [20,26]. At higher concentrations, CXCL12 N-His Tag acts as inhibitor to CXCL12-induced migration (figure 3.5b), suggesting that CXCL12 N-His Tag has a role as an antagonist in CXCR4/CXCL12 signalling axis. At 100 nM, CXCL12 N-His Tag significantly inhibits CXCL12-induced migration of Jurkat cells. Chemokine ligands exist as a mixture of monomeric and higher order species, and the changes in the oligomeric state of CXCL12 dictates the migratory outcome, where monomers, but not dimers, induce cellular migration [324]. Drury *et al.* (2011) has demonstrated that oligomeric states of CXCL12 mediate distinct biological effects through the same receptor, and dimeric CXCL12 exhibits receptor interactions, downstream signalling and migration responses distinct from the monomeric CXCL12 [323]. From the data reported by others [18,325,326], we hypothesise that CXCL12 N-His Tag may have the same function as the dimeric form of CXCL12, where it acts as a partial agonist of

CXCR4, thereby stimulating calcium mobilisation, but showing no chemotactic activity. So, at increasing concentrations, the hypothesis is that it starts to bind to the receptors more readily than the endogenous ligand, and hence causing a significant reduction in CXCL12-induced migration in Jurkat cells [324,325].

These data imply that modification to the CXCL12 by adding 15 amino acids of polyhistidine Tag in N-terminus causes these effects. Crump *et al.* (1997) has shown that modification to Lys-1 and Pro-2 resulted in loss of activity but generates potent SDF-1 antagonists [327]. They have also reported that the first 8 residues of SDF-1 is important for activation. Hence, addition of amino acid residues at the N-terminus of CXCL12 N-His Tag may abrogate the ability of CXCL12 to fully bind to chemokine receptor CXCR4 to induce chemotactic signalling but it can still activate other signalling events such as calcium mobilisation [326]. By shifting the relative positions of N-terminal amino acids of SDF-1, it seems to affect the binding ability of CXCL12 to CXCR4, which results in different signalling events. The interaction between CXCL12 and CXCR4 has been proposed to occur through a two-step process [66,270,328]. The binding between 12-17 residues of CXCL12 and 2-36 residues of CXCR4 results in a conformational change and this change would then facilitate interaction between the first 8 amino acid residues of CXCL12 and a revealed binding pocket in CXCR4, which have been previously discussed as an important interaction for activation [257,327,329].

CXCL12 can be converted into a potent antagonist of CXCR4 by as little as a single N-terminal amino acid substitution (P2G) [327]. Recently, Qin *et al.* (2015) has provided insights into CXCR4 interactions with CXCL12, where the structure revealed that there is a more extensive binding interface than anticipated from the previously described two-site model [330]. Chemokines interact via their globular core with the receptor N-terminus (chemokine recognition site 1) and via their N-terminus with the receptor TM pocket (chemokine recognition site 2) [270]. In complex with vMIP-II, a high affinity antagonist of CXCR4, Qin *et al.* (2015) showed that the binding pocket of CXCR4 is open and negatively charged and can be separated into a major and minor subpocket [330,331]. The binding of chemokine recognition site 1 is dominated by ionic interactions between positively charged residues in the chemokine and negatively charged amino acids at N-terminus and extracellular surface of the receptor, including

sulfonated tyrosines [270,326]. CXCR4 is a rare CXC receptor that possesses a sulfotyrosine in the proximal N-terminus, which may explain its unique ability to engage vMIP-II via its basic N- or β2-β3 loops, which is important for vMIP-II and CXCL12 recognition [330]. While engaging a conserved set of binding determinants, Qin *et al.* (2015) [330] showed that ligands may occupy different regions of the binding pocket, which may explain the ability of CXCL12 N-His Tag binding to CXCR4, but has a different mechanism and acts as an antagonist to CXCR4/CXCL12 binding.

Another possible hypothesis for these results is that CXCL12 N-His Tag may act as a negative allosteric modulator, where it binds in an allosteric site which leads to a conformational change to the receptor CXCR4 and thus, decreasing the affinity for CXCL12 binding at active sites. At higher concentrations, there would be more binding of CXCL12 N-His Tag to the allosteric sites of available CXCR4 in Jurkat cells, and thus causing conformational changes, reducing CXCL12 binding to CXCR4 and migration of Jurkat cells in chemotaxis assays. Allosteric regulation is the process associated to conformational and functional transitions in protein structures by binding of an effector at a site other than the protein's active site [306]. Planesas *et al.* (2015) proposed that CXCR4 has a minimum of 2 allosteric binding sites in distinct domains (figure 3.12) attending to the allosteric functionality, such as antagonists to inhibit inflammation and cancer or agonists to enhance angiogenesis in tissue regeneration [306]. The mechanism of action of allostery is very complex, however, they may represent a novel drug target. Hence, the identification and studying of allosteric residues is useful and important [332–334].

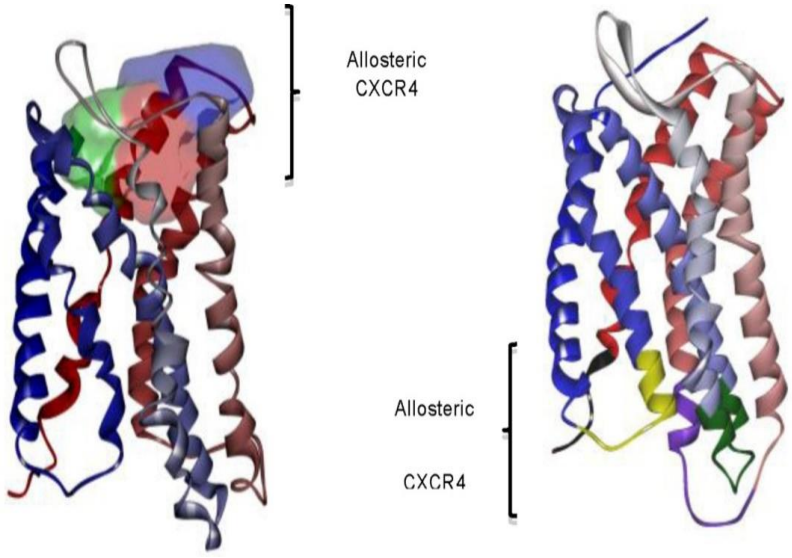


Figure 3.12: Studying the binding interactions in allosteric sites of the CXCR4 receptor. There is a possibility that CXCL12 N-His Tag binds to allosteric sites of CXCR4 causing negative allosteric effects in CXCL12-induced migration via CXCR4 in Jurkat cells [306].

Collins *et al.* (2017) [308] identified that there is a synergistic relationship between CXCL14 and CXCL12 via allosteric modulation of CXCR4. However, the results (figures 3.8 and 3.9) obtained in this chapter contradicts their findings. Contrary to expected increase in CXCL12-induced migration of Jurkat cells by CXCL14, these results showed that CXCL14 significantly decreased the migration of Jurkat cells when treated in combination with 1 nM CXCL12 (figures 3.8 and 3.9). Chemokines, upon activation of their corresponding receptor, induce intracellular signalling cascades that result in cell activation and motility. This includes G-protein-mediated and -independent pathways, such as arrestin-dependent signalling and MAPK/ERK signalling, however, this is dependent on cell-type, ligand-type and receptor-type to generate different intracellular responses [20,312,313]. CXCR4 is widely expressed in primary cells, including tissues and peripheral blood mononuclear cells (PBMCs). Collins and his group isolated T, B and NK cells that uniformly expressed CXCR4 [335]. One of their investigations involved experiments with lymphocytes, where they have demonstrated robust cell migration by combining subactive concentrations (0.1 nM or 1 nM) of CXCL12 with increasing concentrations of CXCL14, which peaked at 300 nM CXCL14. By using CXCR4 antagonist, AMD3100, they managed to show that the synergy response was indeed depend on CXCR4 [308]. Collins and his group concluded that the underlying mechanism of synergism between CXCL14 and CXCL12 correlates with conformational changes induced by CXCL14 and/or dimer formation of cell-surface

CXCR4 [308]. In their studies, they used PBMCs and transfected murine pre-B-cell line 300.19 while in our studies, we used Jurkat cells, human leukemic T-lymphocytes. This may be the possible explanation to this rather contradictory result and showing that this signalling is cell-type specific.

Even though our findings differ from what was reported by Collins *et al.* [308], the results of this chapter are consistent with what was reported by Tanegashima *et al.* (2013) [310]. Tanegashima and his group have demonstrated that CXCR4 is the ligand binding receptor for CXCL14 and that CXCL14 acts as a natural inhibitor of the CXCR4/CXCL12 signalling axis. Since the activity of CXCL14 is analogous to the CXCL12 dimer, it is suggested that the underlying mechanism may be related to dimerization of CXCL14 since there was a complex between CXCL14 and CXCR4 detected. CXCL14 could suppress the tumour growth via specific inhibition of the CXCR4/CXCL12 signalling [310]. Despite the potential clinical significance of CXCL14, it remains unclear whether CXCL14 modulates CXCR4/CXCL12 signalling physiologically.

Despite the potential clinical significance of CXCL14 in various diseases, molecular mechanisms behind physiological functions of CXCL14 are still poorly understood. CXCL14 has been implicated in the maintenance of tissue macrophages, cancer progression and metabolic regulation [336]. CXCL14 possesses chemoattractive activity for activated macrophages, immature dendritic cells and natural kill cells [336]. CXCR4 is the first chemokine receptor identified that binds CXCL14 with high affinity and specificity [310]. In this chapter, we demonstrated that CXCR4 is the ligand binding receptor for CXCL14. However, from observations in figure 3.7, CXCR4 alone is not sufficient for driving the CXCL14-induced chemotaxis, since Jurkat cells, which express CXCR4, showed no response in chemotaxis assays when treated with CXCL14 compared to positive control CXCL12. There are suggestions that CXCL14 receptor and signal transduction pathways may be different from those of conventional CXC-type chemokines [336] and that CXCL14 could be implicated in CXCR4/CXCL12 axis to modulate important physiological processes in immunological development and stem cell maintenance [308,310,337].

Overall, this chapter has shown that MCF-7 and Jurkat cells may be ideal cells for studying CXCR4/CXCL12 signalling axis, and that CXCL12 is the most appropriate

chemokine to study the biology of CXCR4 in downstream signalling cascades leading to migration of cells.

CXCL12 N-His Tag and CXCL14 seem to have an effect in CXC12/CXCR4 signalling axis but further work is required to establish the roles of these chemokines in this signalling network. There is room for further progress in determining the involvement of other chemokines and its receptors and their relationships in modulating physiological processes in development, cell maintenance and immune responses.

Since these findings showed migration of cells in CXCR4/CXCL12 signalling, we would like to study the roles of two proteins that we believe are involved in initiating downstream signalling cascades, leading to migration of cells. The two proteins of interest are Protein Kinase C (PKC) and arrestins, which will be discussed in chapters 4 and 5 (figure 3.12).

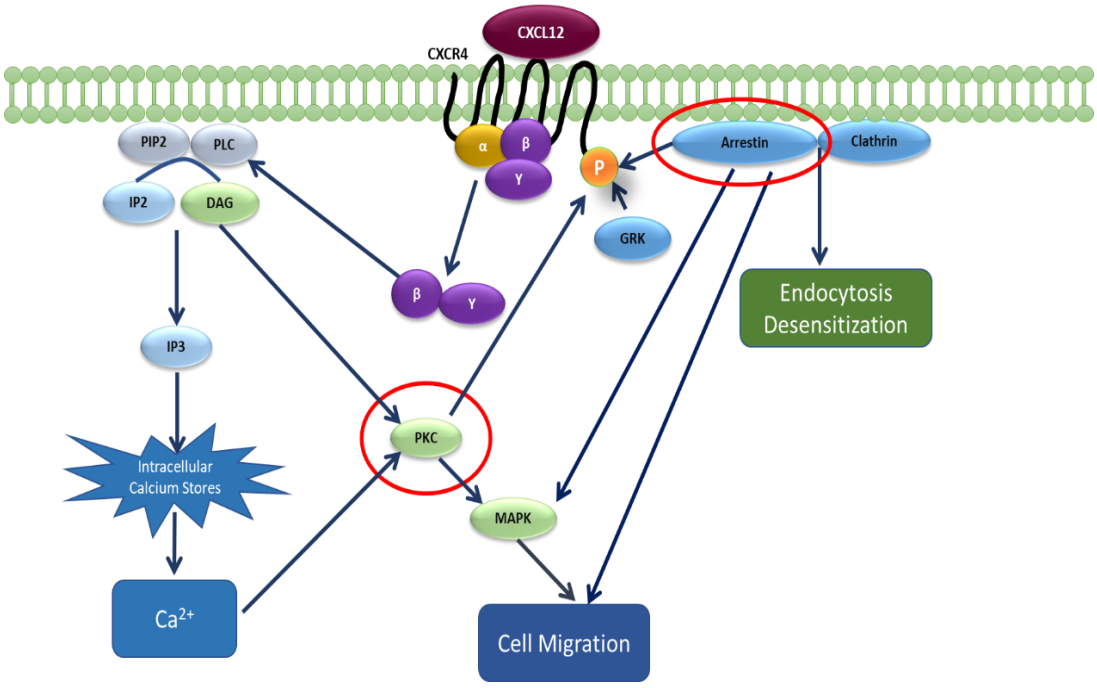


Figure 3.13: A simple schematic overview of chemokine receptor signalling to show proteins that may be involved in migration of cells towards CXCL12. The two proteins of interest, PKC and arrestins have been circled in red.

3.4. Chapter conclusions

The final conclusions to be drawn from this chapter are:

1. CXCR4/CXCL12 signalling axis initiates intracellular calcium signalling in MCF-7 cells and chemotaxis in Jurkat cells.
2. The binding of CXCL12 N-His Tag to CXCR4 induces intracellular calcium signalling in MCF-7 cells but not chemotaxis in Jurkat cells. This indicates the importance of N-terminal residues in CXCL12 in chemotaxis. The data also suggests that there may be allosteric regulation of CXCR4 by CXCL12 N-His Tag.
3. The findings of this chapter suggest that CXCL14 acts as an inhibitor in CXCR4/CXCL12 signalling axis, and these findings were supported by previous studies that showed high affinity binding of CXCL14 to CXCR4 receptors [310,336]. However, these interactions are cell-type specific as there were other studies showing that is synergy relationship between CXCL12 and CXCL14 on CXCR4 receptors in PBMCs [308].
4. Results indicate that MCF-7 and Jurkat cells could be the ideal cells as model systems for studying CXCR4/CXCL12 signalling axis, and CXCL12 is the most appropriate chemokine to study the biology of CXCR4 as investigations with other chemokines did not show any significant results.

CHAPTER 4: Determination of the role of PKC in CXCL12-induced migration of different cancer cell types

4.1. Introduction

Activation of protein kinase C (PKC) is one of the earliest events in the cascade of signal transduction pathways leading to a variety of cellular responses. It has been shown that PKC inhibitors do not affect CCL3-induced cell migration in THP-1 cells [142] but according to recent studies, different chemokine receptor signalling networks are regulated in different ways, in particular, there is a difference in CC- and CXC- receptor families [314]. Some PKC isoforms has also been shown to be implicated directly in migration of cancer cells via chemokine receptors [338–341]. Several groups have shown that receptor desensitisation is not necessarily based only on phosphorylation of agonist- occupied receptors by G-protein coupled receptor kinases (GRK); it can also be caused by phosphorylation of receptors by second messenger-activated kinases such as PKC, to attenuate receptor interaction with G-proteins [66,342]. Oppermann *et al.* (1999) [343] have shown the equivalence in importance of both GRK and second messengers PKC in phosphorylation of CCR5, a chemokine receptor. Second messenger-activated kinases, Protein Kinase A (PKA) and PKC potentially phosphorylate both the ligand bound GPCR and multiple other receptors in a heterologous manner [141]. The complexities and reversals in PKC pharmacology have led to difficulties in understanding the current status of pharmacological activities of PKC [136] and determining whether these observations are receptor- or cell-type specific is not possible from literature alone. For a more in-depth investigation of CXCL12-induced cell migration to be possible, it is important to identify the key points in downstream signalling axis. These points will allow comparisons to be made in CXCL12-induced cell migration of different cancer cell types and hence, drawing a distinct and clearer picture in difference in signal transduction in different cellular background.

4.2. Chapter aims

Hypothesis: We hypothesised that PKC phosphorylation contributes to CXCL12-induced migration in different cell types.

Aims: The primary aim of this chapter is to investigate the molecular mechanisms of CXCL12-induced cell migration, and the importance of PKC activation in this system. This approach should give us a pharmacological insight in the role of PKC in cell migration and also, to verify specific signalling pathways in different cell settings. This will allow future observations to be related to a specific signalling axis and allow comparison to relevant literature.

4.3. Results

4.3.1. PKC inhibition has no effect on the internalisation of CCR5 receptors or actin polymerisation upon CCL3 activation

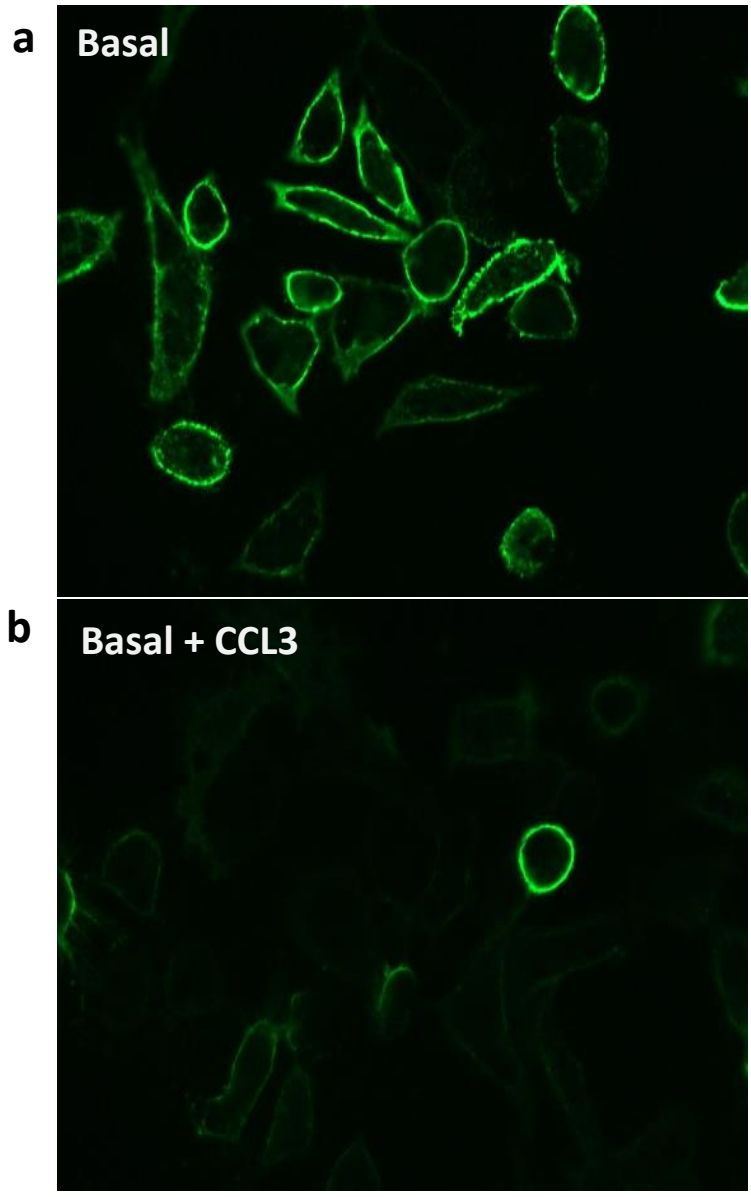


Figure 4.1: CCR5 internalisation in CHO.CCR5 cells upon CCL3 activation. Micrographs of CHO.CCR5 monolayers (a) before and (b) after treatment with 100 nM CCL3. Cells were stained with primary antibody, HEK/1/85a/7a rat monoclonal and secondary antibody anti-rat-FITC and fixed with 4% paraformaldehyde before imaging. Images representative of population. Acquired with Leica imaging suite with 40X magnification.

PKC has been shown to have central roles in signalling in response to many extracellular ligands and can influence many aspects of cell behaviour [344]. As discussed in this chapter's introduction, pan-isotype PKC inhibition does not inhibit migration of THP-1 cells [142], but the involvement of specific isoforms has not been particularly well characterised. Pre-treatment of CHO.CCR5 cells with 5 μM GF109203X and 4 μM Rottlerin for 30 minutes didn't seem to affect CCR5 internalisation upon 100 nM CCL3 activation as loss of CCR5 receptors on the surface (figure 4.2) was observed after incubation of the cells with CCL3 for 30 minutes. Figure 4.2 shows no obvious difference in CCR5 internalisation compared to control experiments (figure 4.1).

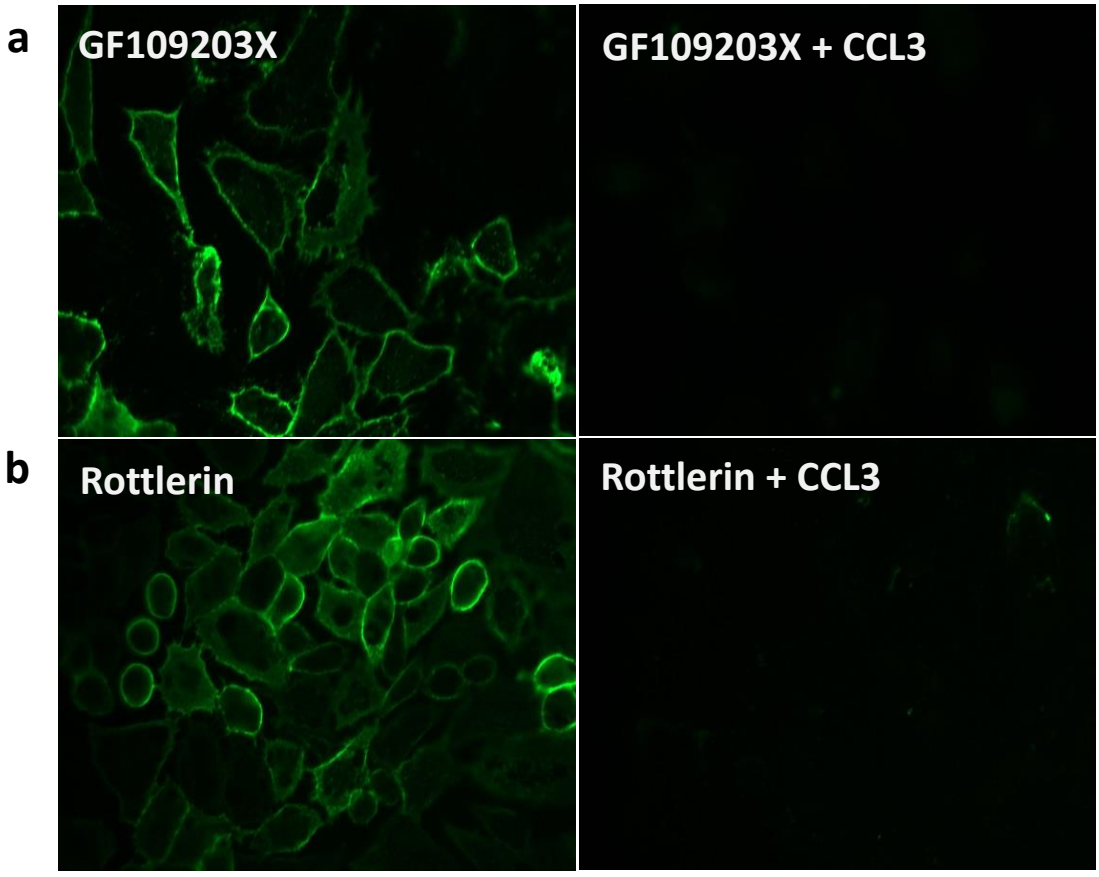


Figure 4.2: PKC inhibitors, GF109203X and Rottlerin, do not affect CCR5 internalisation in CHO.CCR5 cells upon CCL3 activation. Micrographs of CHO.CCR5 monolayers treated with 100 nM CCL3 and PKC inhibitors, (a) GF109203X and (b) Rottlerin. Cells were stained with primary antibody, HEK/1/85a/7a rat monoclonal and secondary antibody FITC and fixed with 4% paraformaldehyde before imaging. Images representative of population. Acquired with Leica imaging suite with 40X magnification.

GF109203X is a potent, highly selective, pan-isotype PKC inhibitor bisindolylmaleimide. GF109203X does not inhibit PKC through the regulatory domain of the kinase and it has an IC_{50} of 0.02 μM. It is a conventional PKC inhibitor that inhibits isoforms α, βI, βII and γ with similar potency [148]. Rottlerin is *Mallotoxin*, which is isolated from *Mallotus philippinesis*. Rottlerin has been widely used as a selective inhibitor of PKCδ at IC_{50} of 3 – 6 μM though its role as a specific PKCδ inhibitor has been questioned. Rottlerin has recently been described as a promiscuous inhibitor, and therefore it is not valid any longer to describe Rottlerin as a selective inhibitor of PKCδ, since there are biological and biochemical processes that are PKCδ-independent that may affect results [152,345].

These results supports the observations made by Cardaba *et al.* [142], which showed that PKC inhibitors do not affect CCL3 induced migration in THP-1 cells. Figure 4.2 showed no effects of CCR5 internalisation upon CCL3 activation, therefore, it is unlikely that the PKC inhibitors will affect CCL3 induced migration via the chemokine receptor CCR5.

Fluorescence microscopy was used to determine if the PKC inhibitors, GF109203X and Rottlerin, disrupt cytoskeleton using Alexa-488 Phalloidin stained CHO.CCR5 cells. CHO.CCR5 cells were used in these experiments, as they are large cell lines and allow for easier identification of cellular structures. These cells were grown as monolayer on coverslips to high confluence (refer to section 2.10.1.; page 60) and were treated for 30 minutes with 5 μM GF109203X or 4 μM Rottlerin and then 100 nM CCL3 for 1 hour prior to fixing and staining. Cells were stimulated with 100 nM CCL3 for 1 hour prior to fixing and staining.

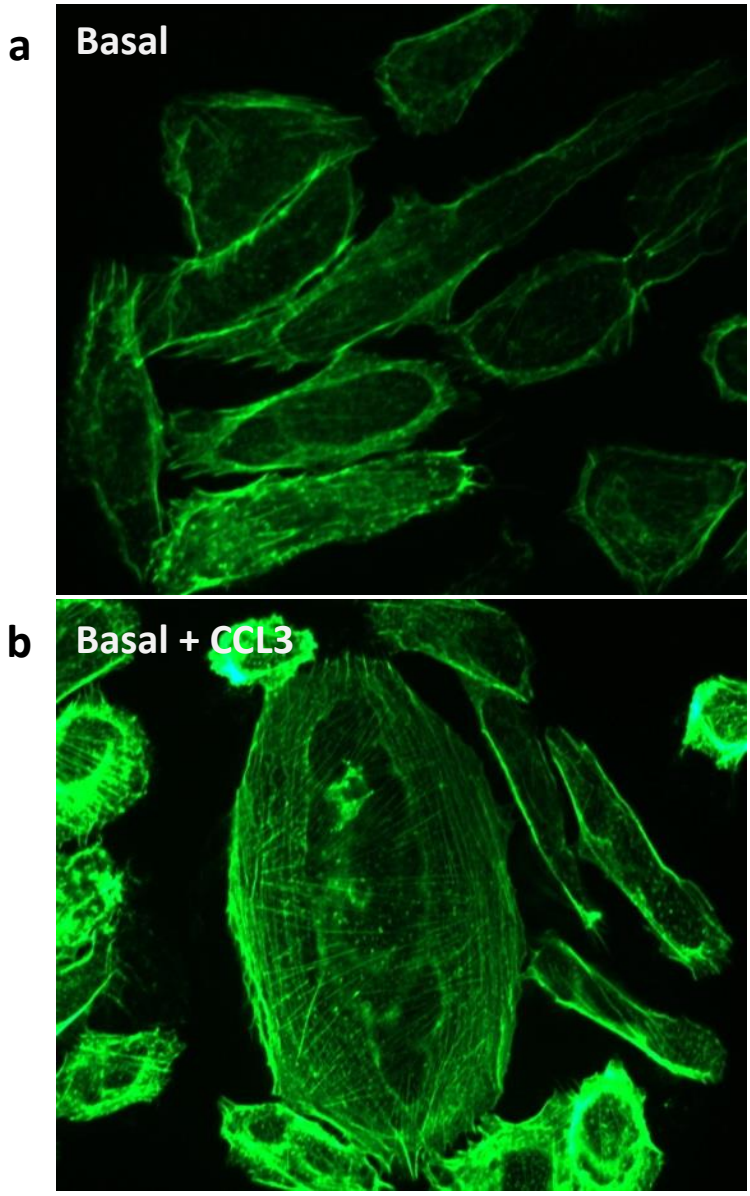


Figure 4.3: Actin polymerisation in CHO.CCR5 cells. Micrographs of CHO.CCR5 monolayers (a) before and (b) after treatment with 100 nM CCL3. Cells were fixed and stained with Alexa-488 Phalloidin actin stain (green). Images representative of population. Acquired with Leica imaging suite with 63X magnification.

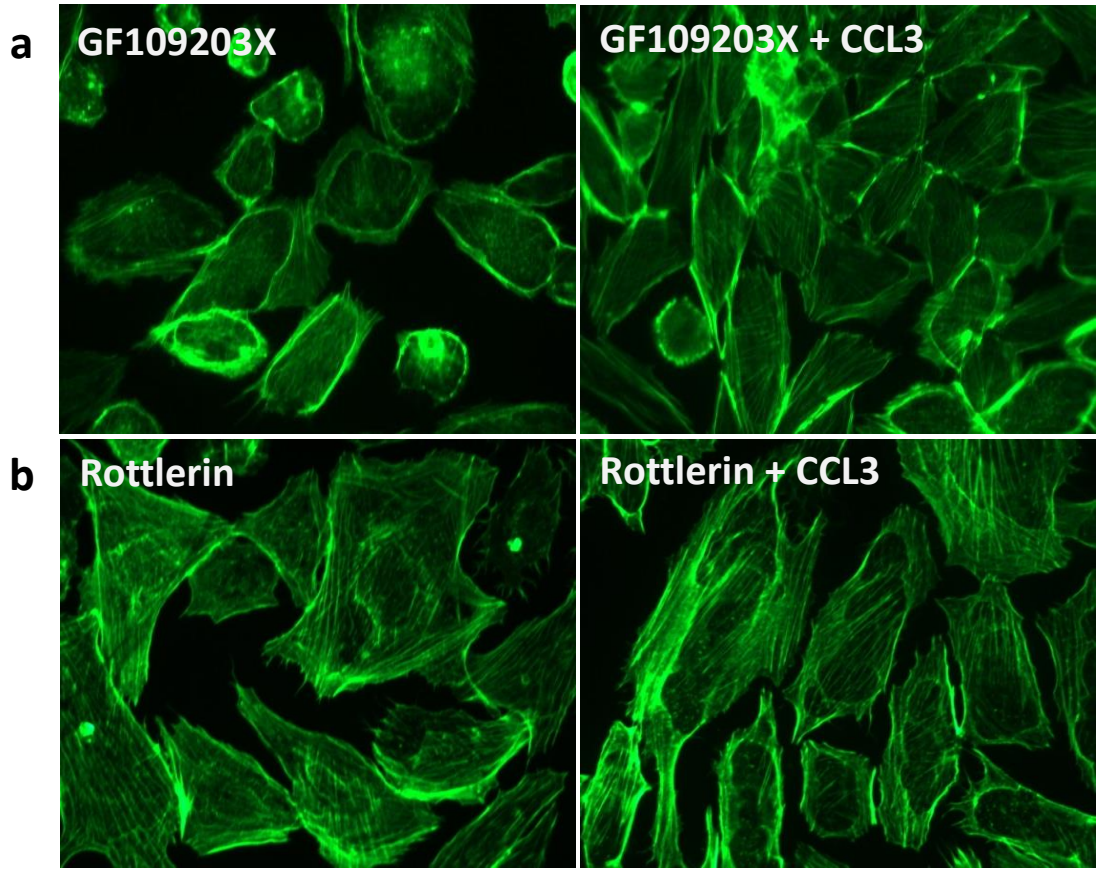


Figure 4.4: PKC inhibitors, GF109203X and Rottlerin, do not disrupt actin polymerisation in CHO.CCR5 cells. Micrographs of CHO.CCR5 monolayers treated with 100 nM CCL3 and PKC inhibitors, (a) GF109203X and (b) Rottlerin. Cells were fixed and stained with Alexa-488 Phalloidin actin stain (green). Images representative of population. Acquired with Leica imaging suite with 63X magnification.

Actin filaments observed were clearly not disrupted in both basal and pre-treated PKC inhibitors CHO.CCR5 cells (figures 4.3, 4.4) and it could be seen that the distribution of actin was uniform. Through fluorescence microscopy, it can be concluded that PKC inhibitors did not disrupt the actin cytoskeleton upon CCL3 activation and the presence of stress fibres were evident.

Alexa-488 Phalloidin binds to actin filaments much more tightly than to actin monomers, and this leads to a decrease in the dissociation rate of actin subunits from filament ends, essentially stabilising actin filaments through the prevention of filament depolymerisation. Upon CCL3 activation, there were visible actin stress fibres observed in stained CHO.CCR5 cells with Alexa-488 tagged phalloidin (figure 4.3). There was no difference observed between CCL3 activated basal CHO.CCR5 and pre-treated PKC

inhibitors CHO.CCR5 cells. These observations agree with results that were published before, where PKC inhibitors do not affect CCL3 induced migration via chemokine receptor CCR5 [142] and unsurprisingly, no obvious disruption of the actin cytoskeleton was observed.

4.3.2. PKC inhibitors do not have any effects on CXCL12-induced calcium release in MCF-7 cells

In the literature, it has been suggested CC- and CXC- chemokine receptor signalling networks are regulated in different ways in cellular migration activation [313,314,346]. As discussed in chapter 3, MCF-7 cells have been widely used in CXCR4/CXCL12 research as they have been shown to highly express receptor CXCR4 when stained with the anti-CXCR4 12G5 mAB [4,8,286]. With that information, it shows us that they are a good model system to study the role for CXCR4 and its signalling axis in the progression to metastasis.

MCF-7 cells were treated with different PKC inhibitors at their working concentrations (refer to table 1.2) to target different PKC isoforms. This would allow the roles of phosphorylation by different PKC isoforms in migration of cells to be assessed individually.

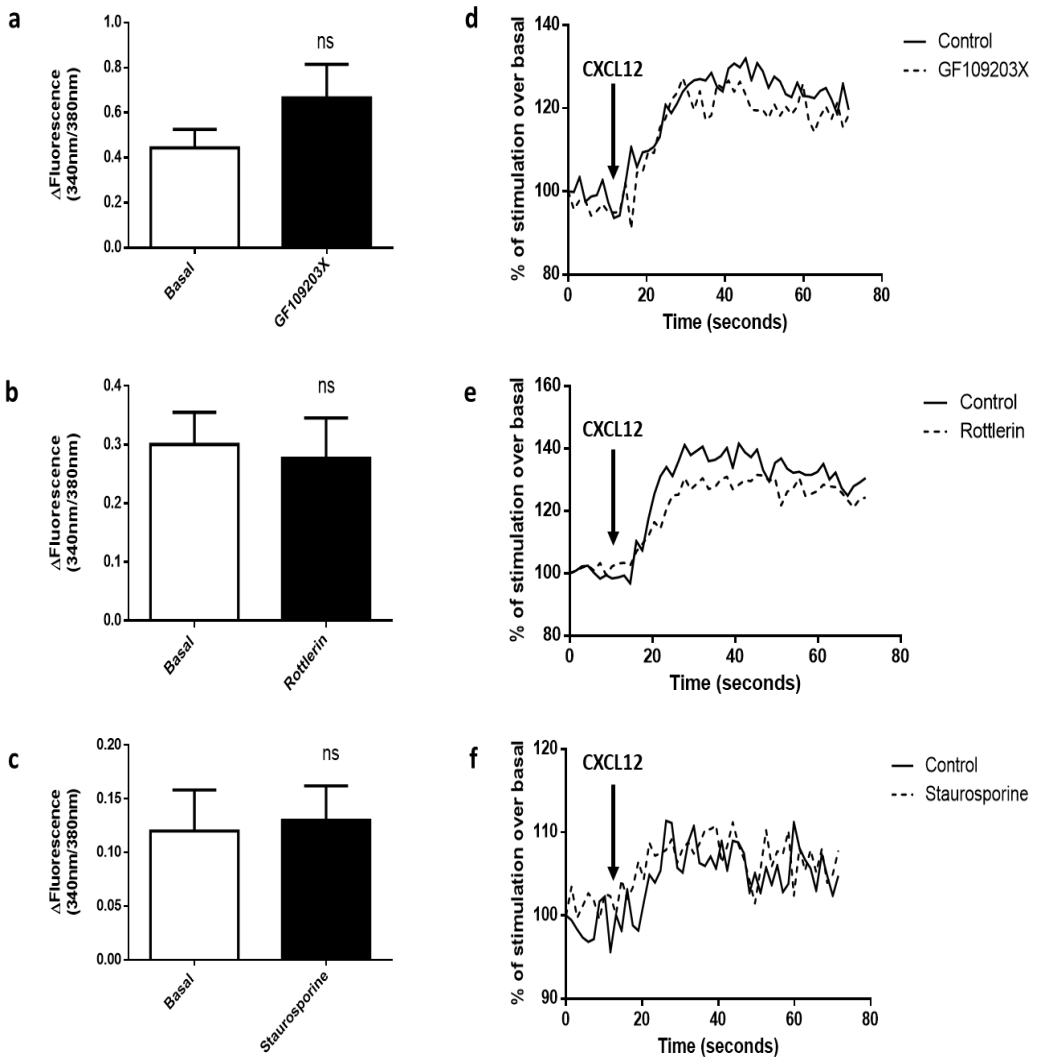
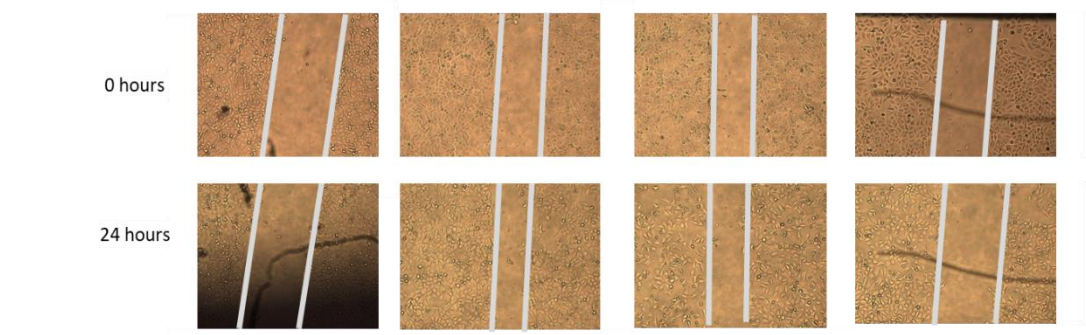


Figure 4.5: PKC inhibitors do not reduce calcium release in MCF-7 cells. (a, b, c) Pre-treatment for 30 minutes with 5 µM GF109203X, 4 µM Rottlerin, 10 nM Staurosporine do not significantly affect intracellular calcium flux in MCF-7 cells after stimulation with 10 nM CXCL12. (d, e, f) Representative tracers showing calcium mobilisation in MC7-cells (10 nM CXCL12). Results represent the mean ± SEM of at least 3 independent experiments (Unpaired t-test, ns = not significant, $p>0.05$)

Pre-treatment of MCF-7 cells with 5 µM GF109203X, 4 µM Rottlerin and 10 nM Staurosporine for 30 minutes had no significant effect on the levels of calcium release observed (figure 4.5). Staurosporine is an antibiotic that is isolated from *Streptomyces staurosporeus.* It is a potent cell-permeable PKC inhibitor and has a similar structure as GF109203X. It has an IC_{50} of 2.7 nM with regards to block the enzyme from rat brain [347].

This indicates that CXCL12-induced calcium release is not PKC-dependent, and since migration of cells can occur independently of intracellular calcium release, migration of cells via CXCR4/CXCL12 signalling axis should be investigated individually to see if PKC activation is needed.

4.3.3. Inhibition of PKC blocks migration of MCF-7 cells in wound healing assays



Mean Basal	+	-	-	-
10 nM CXCL12	-	+	+	+
GF109203X 5 µM	-	-	-	+
Rottlerin 4 µM	-	-	+	-

Figure 4.6: Wound healing assay on MCF-7 cells in the presence or absence of Rottlerin or GF109203X. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. Images are representative of 3 independent experiments.

CXCR4 and its ligand CXCL12 have been shown to play an important role in regulating metastasis of breast cancer to specific organs in the body via a CXCL12-CXCR4 chemotactic gradient [192]. There are a variety of studies that show that PKC isoforms are also important for cancer cell migration [348,349]. Here, we investigated whether PKC is implicated in CXCL12-induced migration in adherent breast cancer MCF-7 cells by blocking the PKC enzymes with the pan-isotype PKC inhibitors, GF109203X, Rottlerin and Staurosporine.

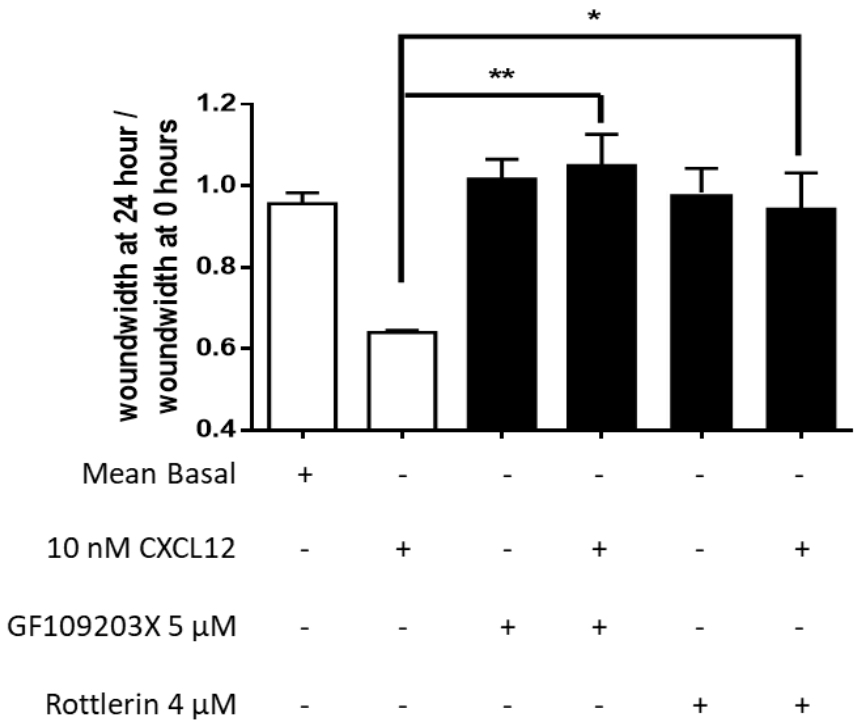


Figure 4.7: Quantification of migration of MCF-7 cells into the wound in the presence or absence of PKC inhibitors, GF109203X or Rottlerin. A value of 1 denotes no migration occurred whereas a value < 1 denotes cell migration. Pre-treatment for 24 hours with PKC inhibitors have significant inhibitory effect on MCF-7 cells migration towards 10 nM CXCL12 vs control in wound healing assays. All results represent the mean ± SEM of 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, *= p≤0.05, **= p≤0.01).

Figure 4.7 showed that PKC may be involved in CXCL12-induced migration in MCF-7 cells as there was a significant inhibition in migration of cells into the wound after 24 hours treatment with PKC inhibitors, GF109203X (**=p≤0.01) and Rottlerin (*=p≤0.05).

For further determination if PKC activation is needed in CXCL12-induced migration in MCF-7 cells, another pan-isotype PKC inhibitor, Staurosporine was used.

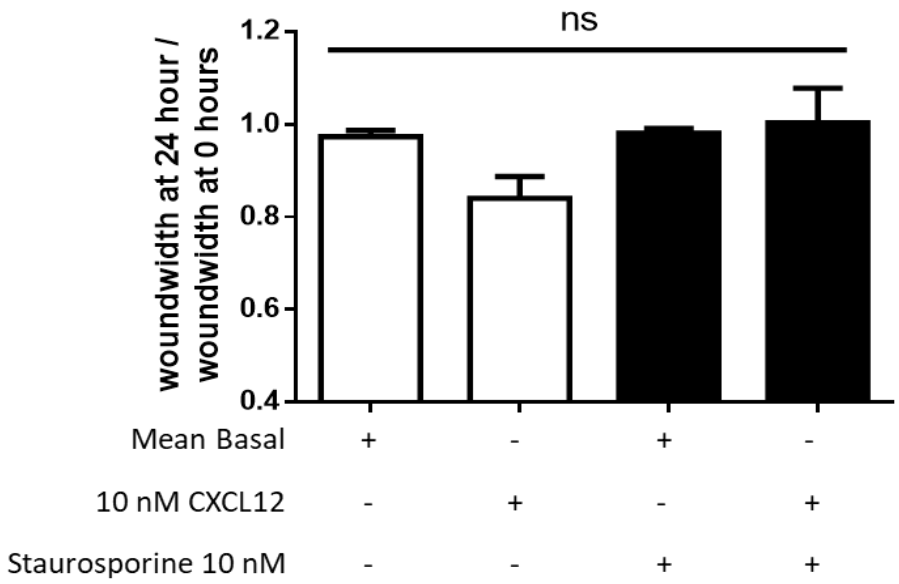


Figure 4.8: Quantification of migration of MCF-7 cells into the wound in the presence or absence of PKC inhibitor, Staurosporine. A value of 1 denotes no migration occurred whereas a value < 1 denotes cell migration. Pre-treatment for 24 hours with PKC inhibitor shows trends of inhibitory effect on MCF-7 cells migration towards 10 nM CXCL12 vs control in wound healing assays. All results represent the mean ± SEM of 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, ns = not significant, p>0.05).

Pre-treatment with Staurosporine had no significant effect but it showed trends of inhibitory effect on MCF-7 cells migration towards 10 nM CXCL12 in wound healing assays (figure 4.8).

These results (figures 4.7, 4.8) show that PKC activation is needed in CXCL12-induced migration of MCF-7 cells via CXCR4. siRNA knockdown of PKCα and PKCζ proteins in MCF-7 cells confirmed the importance of PKC for migration in these cells (figure 4.9), where Mills *et al.* (2016) [286] showed that the loss of conventional isoform, PKCα, and atypical isoform, PKCζ, completely abolishes any migration towards CXCL12.

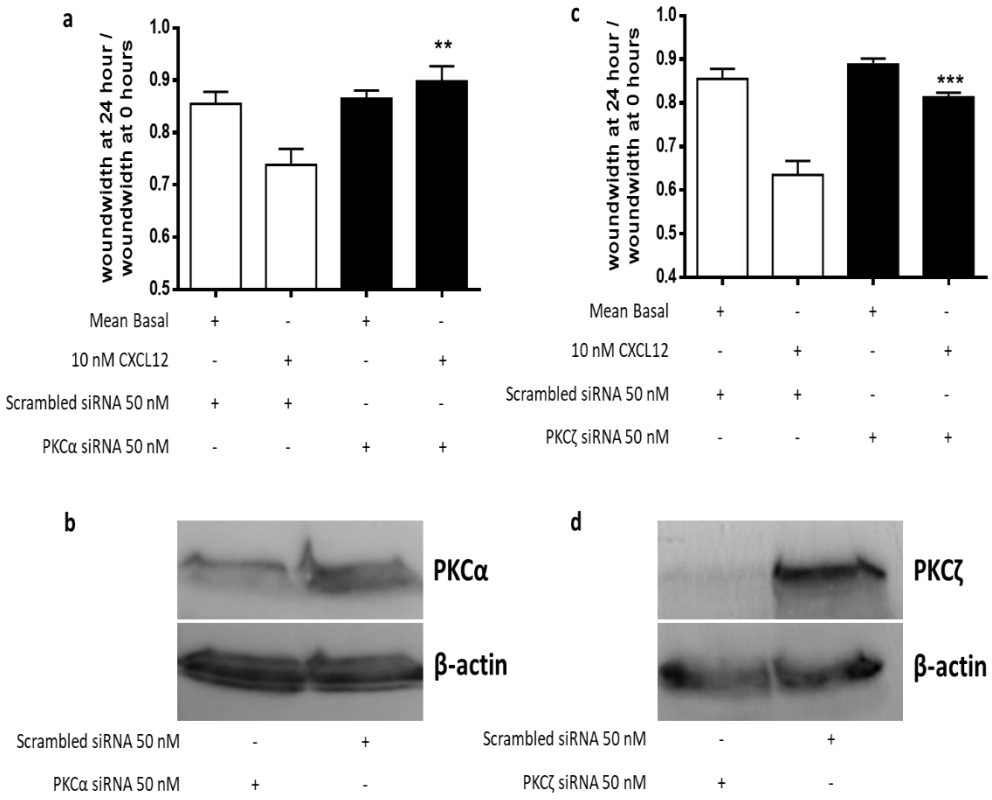


Figure 4.9: PKCα and PKCζ siRNA knockdown experiments showed significant loss of CXCL12-induced migration in chemically transfected MCF-7 cells. (a) Wound healing assay on MCF-7 cells after transfection with 50 nM scrambled siRNA as control or PKCα siRNA. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. (b) Western blot analysis of PKCα expression in MCF-7 cells after siRNA knockdown, where β-actin acts as loading control. (c) Wound healing assay on MCF-7 cells after transfection with 50 nM scrambled siRNA as control or PKCζ siRNA. Cell migration was induced with 10 nM CXCL12 and measured after 24 hours. (d) Western blot analysis of PKCζ expression in MCF-7 cells after siRNA knockdown, where β-actin acts as loading control. Quantification of migration of cells into the wound. A value of 1 denotes no migration occurred whereas a value < 1 denotes cell migration. All results represent the mean ± SEM of 6 independent experiments (One-way ANOVA, Bonferroni multiple comparison, **= p≤0.01, ***= p≤0.001). Note: Collaboration with Shirley Mills in the laboratory [286].

MCF-7 cells were chemically transfected with scrambled siRNA as control, PKCα or PKCζ siRNA via Turbofect for 24 hours. Transfected MCF-7 cells were treated with 10 nM CXCL12 for another 24 hours before wound healing assay analysis (refer to section 2.9.3.; page 59). There is a significant loss of migration in chemically transfected PKCα and PKCζ siRNA MCF-7 cells, and therefore, confirms the importance of PKC activation for migration in these cells (figures 4.9a and 4.9c). The success of knockdown was confirmed by western blot analysis as shown in figures 4.9b and 4.9d.

4.3.4. Inhibition of PKC has no effect on Jurkat cell migration

As discussed in this chapter's introduction, pan-isotype PKC inhibition does not inhibit migration of THP-1 cells [142], but the involvement of specific isoforms has not been well characterised particularly as there is some evidence that atypical PKCζ is directly involved in CXCL12 signalling in immature human $CD34^{+}$- enriched cells and in leukemic pre-B Acute Lymphocytic Leukaemia (ALL) G2 cells [339].

As further confirmation to see if PKC activation is needed in CXCL12-induced migration, leukemic Jurkat cells were used as they have been shown to express CXCR4 [286] and they are good model system that are frequently used to study CXCR4/CXCL12 signalling axis.

CXCL12-induced cell migration depends on certain intracellular proteins, which need to be activated to allow the cell to move towards the chemokine stimulus [11], hence it is important to study different signalling cascades and see whether they are implicated in CXCR4 migration in suspension cells compared to adherent cells.

Pre-treatment of suspension leukemic T-lymphocytes, Jurkat cells, with different PKC inhibitors have shown that they have no significant inhibitory effects in chemotaxis towards 1 nM CXCL12 in chemotaxis assays (figure 4.10).

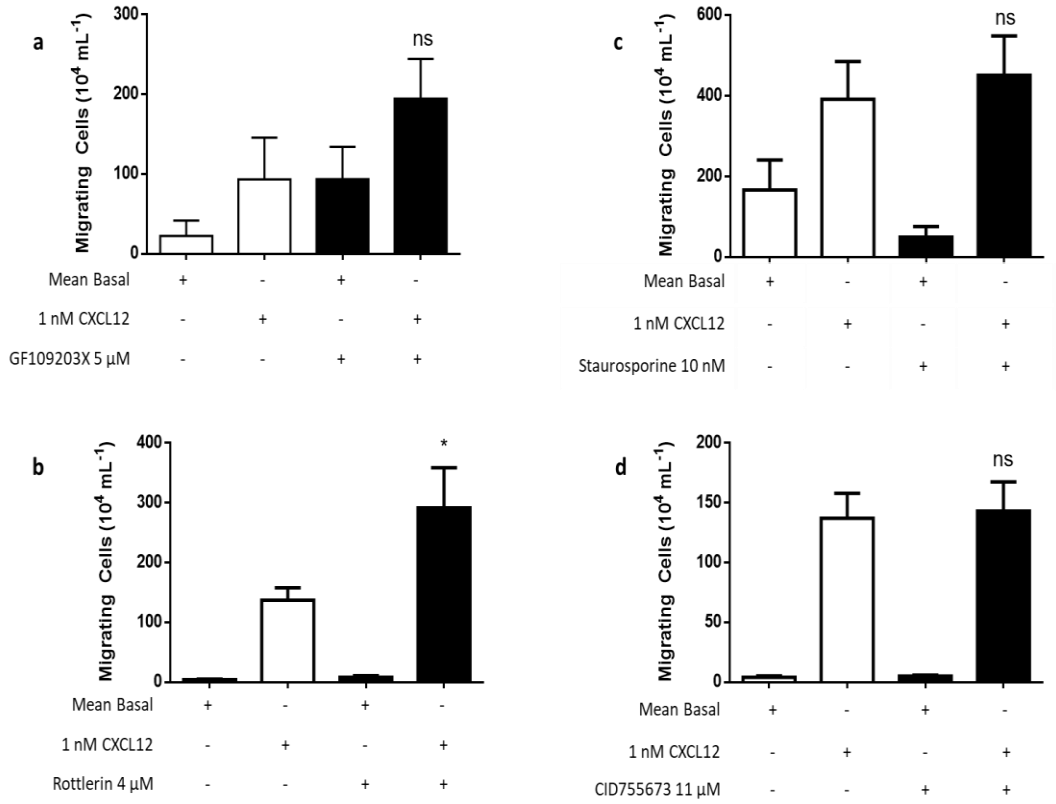


Figure 4.10: PKC and PKD inhibitors have no significant inhibitory effect on CXCL12-induced migration in suspension Jurkat cells. (a) Cell migration towards 1 nM CXCL12 in untreated control cells or GF109203X pre-treated cells. (b) Cell migration towards 1 nM CXCL12 in untreated control cells or Rottlerin pre-treated cells. (c) Cell migration towards 1 nM CXCL12 in untreated control cells or Staurosporine pre-treated cells. (d) Cell migration towards 1 nM CXCL12 in untreated control cells or CID755673 pre-treated cells. All results represent the mean ± SEM of 4 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * = p≤ 0.05, ns = not significant).

This observation is different from what was seen in adherent MCF-7 cells but agrees with the results that were seen in CCL3 induced migration of suspension cell line THP-1, where PKC activation is not essential [142]. Pan-isotype GF109203X, Rottlerin and Staurosporine not only did not have inhibitory effects in migration of cells but showed trends of increasing number of cells migrating (figure 4.10a, b, c). Pre-treatment of Jurkat cells with Rottlerin showed significant increased migration of Jurkat cells (figure 4.10b).

Pre-treatment of Jurkat cells with 5µM GF109203X showed high number of cells migrating, even in mean basal control GF109203X. This suggests that GF109203X might have directly or indirectly induced cell activation, which leads to cell migration.

However, these observations contradict with studies from other groups, as they have shown that GF109203X is a potent and selective inhibitor of PKC [138,148]. Alternatively, PKC has a role in receptor desensitisation, and hence slowing or stopping basal cell migration. In these experiments, activation of PKC was inhibited by GF109203X. This might cause reduced activation of PKC, resulting in continued activation of receptor and therefore, migration of cells.

To confirm that PKC activation is not essential in CXCL12-induced migration in Jurkat cells, a more specific protein kinase D (PKD) inhibitor, CID755673, was used. PKD is a serine/threonine kinase with three isoforms; PKD1-3 [350]. It was previously thought to be PKC isoform, PKCμ, but catalytic domain homology has since distinguished PKD as a member of calcium calmodulin-dependent kinase (CaMK) family [154]. Figure 4.10d shows that there was no inhibition in migration of Jurkat cells when cells were treated with CID755673.

siRNA knockdown experiments were also undertaken to confirm the importance of PKC for CXCL12-induced migration in Jurkat cells.

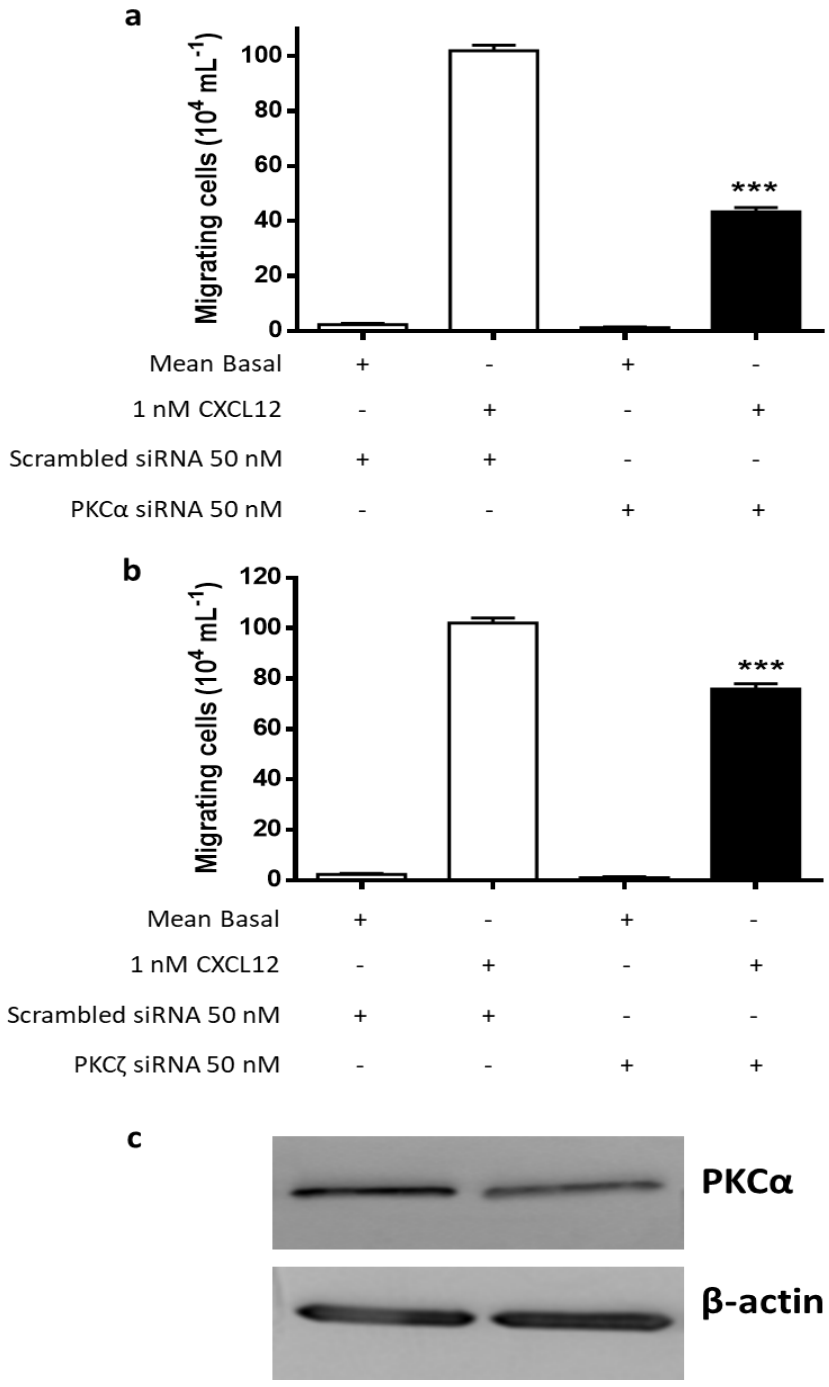


Figure 4.11: PKCα and PKCζ siRNA knockdown experiments showed significant loss of CXCL12-induced migration in electroporation transfected Jurkat cells. (a) Cell migration towards 1 nM CXCL12 in Jurkat cells after transfection with scrambled siRNA as control or PKCα siRNA. (b) Cell migration towards 1 nM CXCL12 in Jurkat cells after transfection with scrambled siRNA as control or PKCζ siRNA. (c) Western blot analysis of PKCα expression in Jurkat cells after siRNA knockdown, where β-actin acts as loading control. All results represent the mean ± SEM of 6 independent experiments (One-way ANOVA, Bonferroni multiple comparison, ***= p≤0.001). Note: Collaboration with Shirley Mills in laboratory [286].

Jurkat cells were transfected with scrambled siRNA as control, PKCα or PKCζ siRNA by electroporation transfection using AMAXA Nucleofector for 24 hours prior to chemotaxis assays (refer to section 2.6.2.; page 55). siRNA knockdown of PKCα and PKCζ proteins in Jurkat cells allow us to differentiate between the use of different PKC isoforms. PKCα knockdown leads to a significant loss of about half the migratory response (figure 4.11a), whereas PKCζ knockdown has less, but still significant, impact (figure 4.11b). In figures 4.11a and 4.11b, there are still a robust number of cells migrating but when compared to migration of control cells (transfected with 50 nM scrambled siRNA), the migration of PKCα and PKCζ siRNA transfected Jurkat cells is significantly lowered. The success of knockdown was confirmed by Western blot analysis (figure 4.11c).

4.4. Discussion

The aim of this chapter was to determine the role of PKC in CXCL12-induced migration of different cancer type of cells through different assays.

PKC is a family of protein kinases enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups or serine and threonine residues on these proteins. PKC isoforms transduce a wide range of extracellular signals that result in the generation of DAG, therefore regulating diverse cellular behaviours such as survival, growth and proliferation, migration and apoptosis [344]. Hence, it is important to investigate the downstream signalling cascades that leads to migration of cells after PKC activation as dysregulation can be associated with a plethora of pathophysiologies [136]. PKCs were famously discovered and studied due to its role as direct signal transducers for phorbol esters [351], a class of plant-derived tumour-promoting compounds, which potently mimics the function of endogenous ligand, DAG [134].

As discussed in introduction, Oppermann *et al.* (1999) [343] have shown that phosphorylation of the receptors by GRK and PKC are both equally important in activating downstream signalling cascades upon ligand activation but the involvement of specific isoforms has not been well characterised.

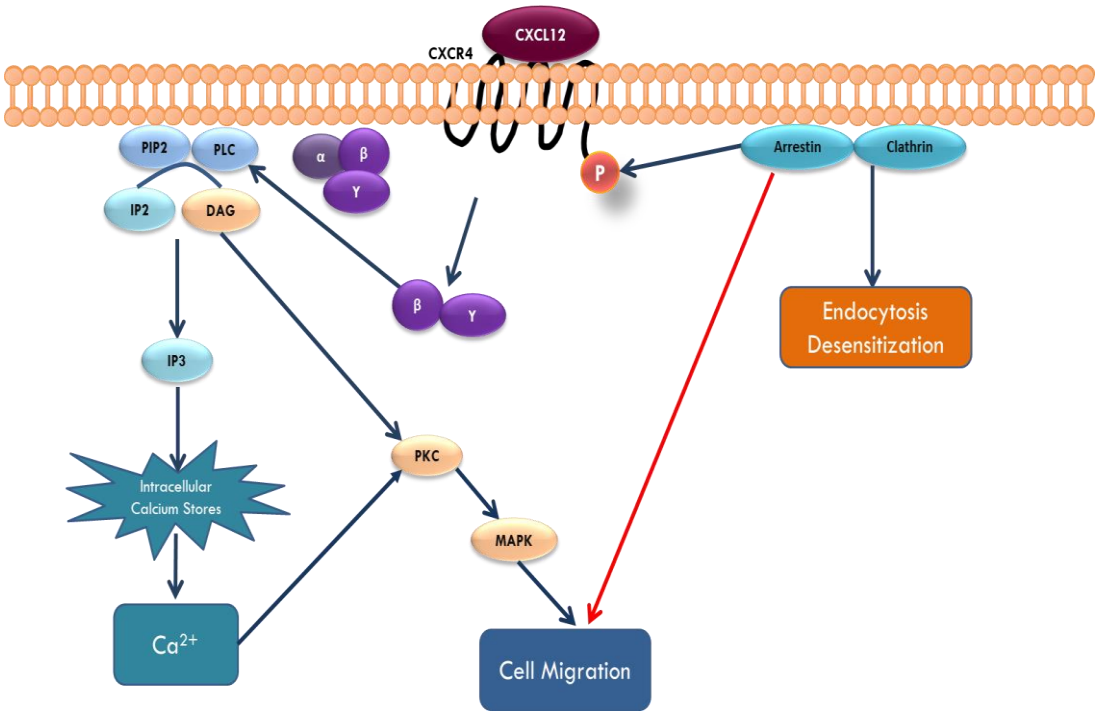


Figure 4.12: Overview of signalling cascade. Schematic overview of signalling cascades involved in the migration of cells towards CXCL12.

Figure 4.12 shows an overview of cascade, and the potential intracellular signalling proteins that are involved in CXCR4/CXCL12 signalling axis. The chemokine receptor CXCR4 has been of interest for a number of years, as it has been shown to be up-regulated in several cancers and its activation can lead to cancer cell metastasis [192,286,352]. Although numerous studies have investigated cell migration of cells over the years, there are still questions that remain unanswered as to which downstream effector proteins are important to contribute in the movement of cells. Furthermore, there is conflicting evidence in the literature about the importance or involvement of downstream signalling partners in different systems [78,338,339,353]. Characterisation of the signalling molecules is usually carried out specifically. Since there are so many different cell types or read out systems and approaches, it is unsurprising that some of the data may contradict each other. Therefore, studying the main signalling molecules that are thought to be of importance in CXCL12-induced migration in adherent cells versus suspension cells can give us a clearer picture of CXC-system in different cell types.

One of the findings in this chapter is that there are indeed differences in which signalling molecules are important for cell migration in leukemic cells compared to

adherent breast cancer cells. Both Jurkat and MCF-7 cells do express CXCR4 and migrate towards CXCL12, however the signalling molecules between the two cell types show some intriguing differences [286].

Small molecule inhibitors were used in experiments to investigate the importance of PKC activation in migration of cells. Studies have shown that all PKC inhibitors possess some degree of promiscuity, but conventional and novel PKCs have been shown to somewhat have lower levels of selectivity, with approximately 5-10% of compounds in screen hitting their isoforms [231,354–356].

There are a variety of studies that show that PKC isoforms are also important for cancer cell migration [341,348]. Pan-isotype inhibitors, GF109203X and Rottlerin, completely blocks breast cancer cell migration towards CXCL12 (figures 4.7); however, at the same concentration of the inhibitors, there is no inhibitory effects on the migration of the leukemic suspension cells towards CXCL12 (figures 4.10a, 4.10b). Instead, it seems like GF109203X and Rottlerin lead to an increase in migration of the suspension Jurkat cells (figures 4.10a, 4.10b). This result agrees with previous studies, where PKC inhibitors do not block the migration of suspension THP-1 cells [314], as discussed above.

To put into context, PKC activation may be required to activate MAPK-induced migration in adherent MCF-7 cells but in suspension Jurkat cells, it is implied that PKC may have a role in desensitisation of CXCR4 receptor. If PKC activation is inhibited and resulting in absence of phosphorylation via PKC, signal termination may not be able to occur. Hence, it can be hypothesised that the increase in CXCL12-induced migration of Jurkat cells is due to PKC causing a prolonged desensitisation of the receptor. These observations suggest that there are differential effects of PKC-mediated desensitisation in different cell types, which leads to different cellular responses.

siRNA approach was used to further confirm the results obtained with the small molecule inhibitors and it showed that knockdowns of PKCα and PKCζ in MCF-7 cells completely blocks movement of the cells in wound healing assays (figures 4.9a, 4.9c) [286], confirming the results acquired from inhibition of PKC using small molecule antagonists (figures 4.7, 4.8). However, knockdown of these PKC isoforms in suspension Jurkat cells generated a different and more complicated picture as knockdowns of PKCα and PKCζ has contradicted with observations with small

molecule antagonists. Knockdown of PKCα and PKCζ result in a significant loss of migratory cells (figures 4.11a, 4.11b), which is very different from the observations seen in PKC inhibition studies with small molecular antagonists (figure 4.10). Hence, it is speculated that there is a difference in the usage of PKC in CXCL12-induced migration of these two cell types [286].

As discussed previously, the structure of all PKCs consists of a regulatory domain and a catalytic (also known as kinase) domain, which are joined together by a hinge region [357]. The catalytic region is highly conserved among the different isoforms, and this is the region that allows for different functions to be processed [131]. Another feature of the PKC catalytic region that is essential to the viability of the kinase is its phosphorylation. The conventional and novel PKCs have three phosphorylation sites; the activation loop, the turn motif and the hydrophobic motif; while atypical PKCs are phosphorylated only on the activation loop and the turn motif [357]. These phosphorylation events are essential for the activity of the enzyme, except for phosphorylation of the hydrophobic Ser660/Thr729 motif as it has been shown to be unnecessary by the presence of glutamic acid which acts as a negative charge and similar to a phosphorylated residue [130,358,359].

Based on the observations from these experiments, it can be speculated that MCF-7 cells need the catalytic activity of PKC and so small molecule antagonists as well as knockdown prevents migration of cells, whereas Jurkat cells may not need the kinase activity of PKC, which is implicated in cell migration, but it may depend on the functionality of the other PKC isoforms domains. It has been shown that PKCs can be cleaved by caspases, generating a catalytically active kinase domain and a freed regulatory domain fragment that can act both as an inhibitor of the full-length enzyme and as an activator of certain signalling responses [132].

Overall, the data in Jurkat cells is more complex, but they show a difference towards use of PKC in MCF-7 cells and so further investigations should be carried out. To draw a clearer picture, it is important to investigate downstream signalling cascades following phosphorylation of receptors. Arrestins, another group of proteins that have been known to play a role in desensitisation and migration of cells, bind to phosphorylated receptor. Hence, studying arrestins is the way forward to further

characterise the role of PKC and, to understand the role of arrestins in different cell types as difference in usage of PKC in two different cell systems was observed.

4.5. Chapter conclusions

The final conclusions to be drawn from this chapter are:

1. PKC activation is essential in adherent breast cancer cells, MCF-7 cells as inhibition of PKC blocks MCF-7 cell migration. This result was further confirmed by siRNA knockdown experiments, where it was shown that knockdown of conventional PKCα and atypical PKCζ, abrogates movement of cells.
2. Inhibition of PKC has no inhibitory effects on suspension leukemic Jurkat cells migration but knockdown of PKCα and PKCζ result in a significant loss of migratory cells. This suggests that there is a difference in the usage of PKC isoforms domains in different cell types.
3. This chapter concludes that the cellular background may be important for distinct signalling pathways used by the CXCR4 receptor and therefore a generalisation of how CXCR4 induces migration in different cell types and species should be avoided.

CHAPTER 5: Role of arrestins in CXCR4/CXCL12 signalling axis

5.1. Introduction

Over the past decade, significant efforts have been made to unravel the regulatory properties and molecular mechanisms that control arrestins' interactions with GPCRs. One of arrestins' roles involve selectively binding to the active phosphorylated forms of their cognate GPCRs, which mediate receptor desensitisation by blocking G-protein coupling [163]. In addition to their 'classical' function in desensitisation and internalisation, arrestins are important for regulating GPCR signal transduction [108,109]. Currently, there are four known arrestin subtypes; arrestins 1 and 4 have been shown to be expressed in photoreceptor cells and are known as the two visual subtypes while arrestins 2 and 3 (also known as β-arrestins-1 and -2) are non-visual arrestins, which are expressed ubiquitously in all cells and tissues. Arrestins 2 and 3 not only have a function in the desensitisation of most GPCRs, but they also serve as multifunctional adaptors and scaffolds of GPCR signalling [80,81]. All these insights have shed some light on the complexity involving GPCR-arrestin interactions and to help researchers understand arrestins' role in development of cancer and tumour metastasis through chemotaxis [81,171]. Over the years, various signalling molecules that are involved in CXCR4 triggered migration have been identified, however, there is still some uncertainty about which pathways are directly involved in cell migration. The requirement of arrestins for chemotaxis in the downstream signalling networks of multiple receptors implicates that arrestins play a key role in mediating cell migration [81,246,360]. Studies on chemokine receptors suggest that receptor desensitisation and recycling are important for cell polarisation during chemotaxis [80,81]. Furthermore, arrestins facilitate the activation of numerous effector pathways, such as MAPKs and Akt, and acts as a scaffold for specific components of the MAPK signalling cascade [185,361]. As mediators of receptor desensitisation, arrestins contribute to cell polarity during chemotaxis by quenching the signal at the trailing edge of the cell. An alternative theory is that arrestins scaffold signalling molecules that are involved in cytoskeletal reorganisation to promote localised actin assembly events leading to the formation of leading edge [362–365]. The involvement of arrestins in chemokine release as well as actin reorganisation suggests that they play multiple roles in the *in vivo* chemotactic process. More extensive studies that evaluate the speed and distance

of migration, as well as more *in vivo* studies that demonstrate immune cell infiltration, cancer metastasis, wound healing, and other cellular responses, are necessary to fully elucidate the role of each pathway [183].

5.2. Chapter aims

Hypothesis: We hypothesised that arrestins is important in CXCL12-induced migration in different cell types.

Aims: The primary aim of this chapter is to investigate the role and importance of arrestin 2 and arrestin 3 in CXCL12-induced migration and whether they are involved either in cell polarisation or act as potential scaffolds in this system. This approach should give us some pharmacological insights into the role of arrestins in cell migration and help us to understand the interactions of arrestins with different signalling molecules, such as PKC, within different cellular settings. This will allow future observations to be related to a specific signalling axis and allow comparison to relevant literatures.

5.3. Results

5.3.1. Investigation of the role of arrestins in non-invasive adherent breast cancer cells, MCF-7 cells via CXCR4 upon CXCL12 activation

Recent findings indicate that most signalling pathways do not function in isolation, but rather as components of larger signalling networks. It is no surprise that several recent studies showed that arrestins mediate various forms of crosstalk between receptor signalling systems due to arrestins' position in cells as discussed previously [81,261,366]. However, while most studies have focused on roles of arrestins at the plasma membrane and in the cytoplasm, emerging evidence suggests additional functions in the nucleus, specifically in regulating transcription [366]. Both arrestins contain nuclear localisation signals, but only arrestin 3 contains a nuclear export signal. Thus, whereas arrestin 3 is found exclusively in the cytoplasm, arrestin 2 is present in both cytoplasm and the nucleus [193].

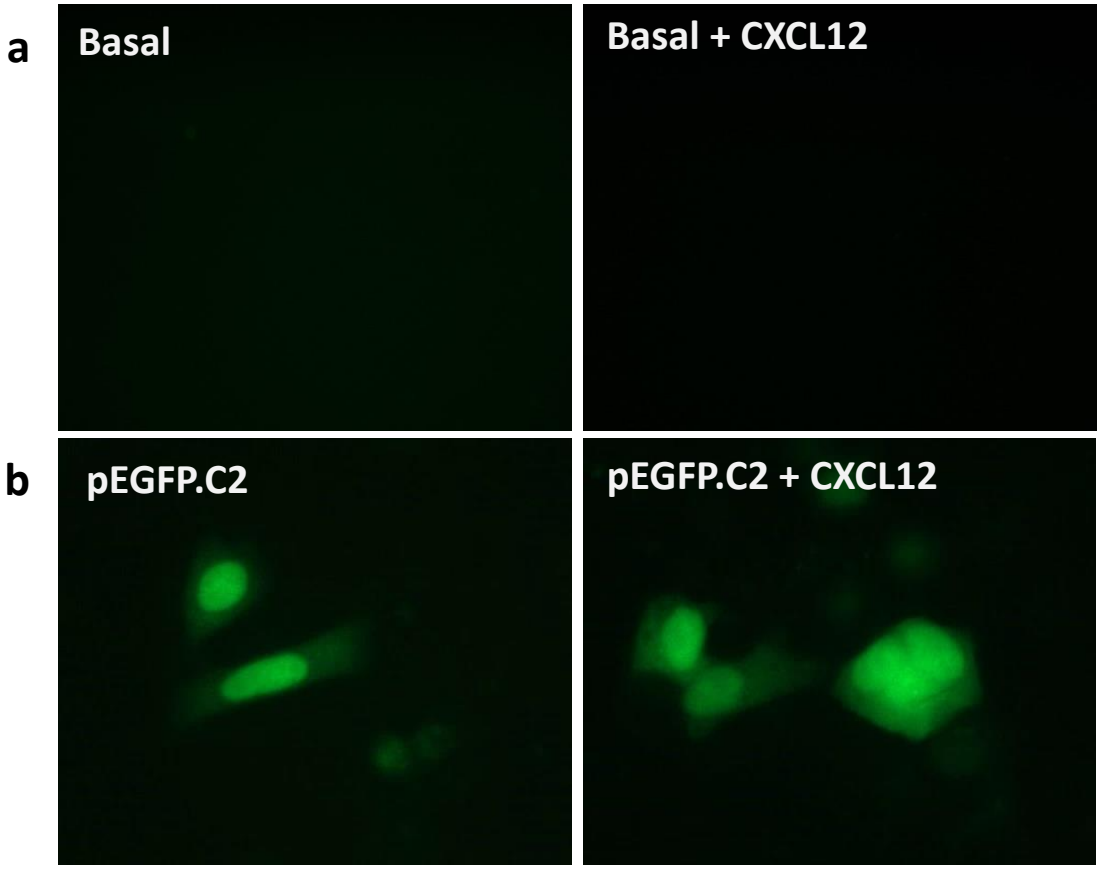


Figure 5.1: Micrographs of MCF-7 monolayers treated with 10 nM CXCL12. MCF-7 cells were (a) mock transfected (basal) as negative control and (b) chemically transfected with 2 µg plasmid DNA coding non-functional pEGFP.C2 as a control for 24 hours. Cells were fixed with 4% paraformaldehyde before imaging. Images representative of population. Acquired with Leica imaging suite with 63X magnification.

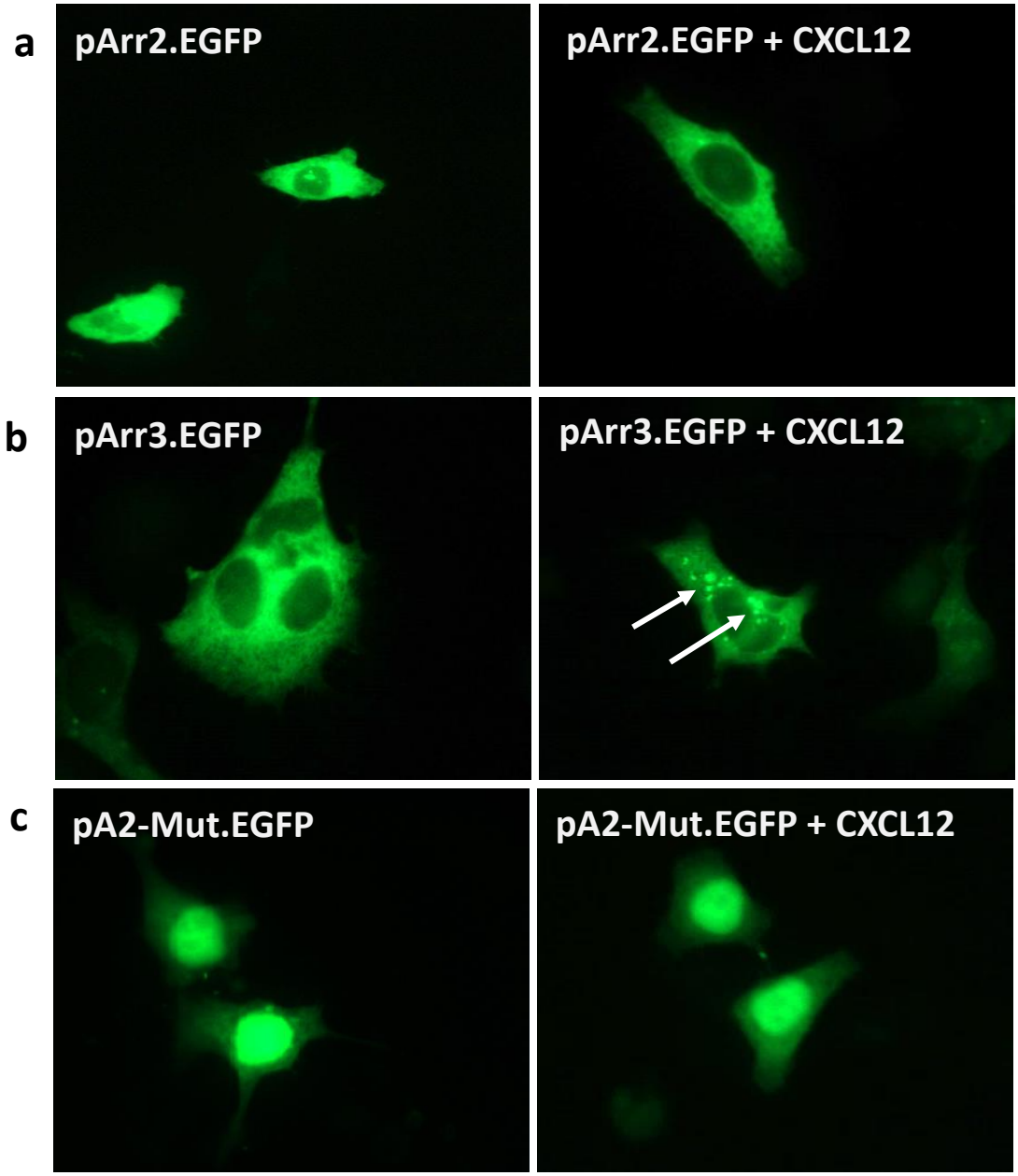


Figure 5.2: Overexpression of arrestins in MCF-7 cells upon CXCL12 activation. Micrographs of MCF-7 monolayers treated with 10 nM CXCL12. MCF-7 cells were chemically transfected with 2 µg plasmid DNA coding for (a) EGFP tagged arrestin 2 (pArr2.EGFP), (b) EGFP tagged arrestin 3 (pArr3.EGFP) and (c) EGFP tagged arrestin 2-mutant (pA2-Mut.EGFP), for 24 hours. Cells were fixed with 4% paraformaldehyde before imaging. Images representative of population. Acquired with Leica imaging suite with 63X magnification.

Figure 5.1 shows control experiments and figure 5.2 shows successful transient transfection of EGFP tagged arrestins 2,3 and arrestin 2-mutant in MCF-7 cells. Overexpression of arrestins 2 and 3 in transfected pArr2.EGFP and pArr3.EGFP MCF-7 cells can be found mainly in the cytoplasm. There is a distinct difference in terms of

localisation of these proteins compared with the pA2-Mut.EGFP transfected MCF-7 cells, where the mutated arrestins 2 found spread between the cytoplasm and nucleus of the cells but mostly located within the nucleus of the cell. It appears that point mutation of the protein arrestin 2 changes the location of its accumulation within the cell. This is different when compared to wildtype (WT) arrestin 2, where it can be observed that most of the arrestins were located in the cytoplasm of MCF-7 cells (figure 5.2).

These results (figures 5.1, 5.2) also show the differences in movement of arrestins upon CXCL12 activation via CXCR4 receptors in MCF-7 cells. Arrestins are well known negative regulators of GPCR signalling [366]. Upon GPCR activation, arrestins have been shown to translocate to the cell membrane and bind to the phosphorylated agonist-occupied receptors which will lead to uncoupling of these receptors from G-proteins and promote the internalisation of these receptors, which attenuates signalling [367]. In MCF-7 cells that have been transiently transfected with pArr3.EGFP plasmids, a clear movement of arrestin upon CXCL12 activation is visible, compared to cells which were transfected with pArr2.EGFP and pA2-Mut.EGFP plasmids (figure 5.2). This movement leads to visible change of fluorescence localisation in the cell, where arrestin 3 has clustered together in defined little spots after CXCL12 treatment, showing that arrestin 3 may be involved in CXCR4 activation.

The binding of CXCL12 to CXCR4 induces intracellular signalling through several divergent pathways, which leads to downstream events such as chemotaxis, cell survival, and/or cell proliferation, increase in intracellular calcium and gene transcription [2]. Ligand stimulation can result in an increase in intracellular calcium and this change is usually used as a measure of the chemokine activity [2,142].

Non-visual, arrestins 2 and 3, are known to function either as terminators of receptor signalling and clathrin adaptor proteins by mediating GPCR desensitisation and internalisation, or independently as scaffolds for signalling, where they act as multifunctional adaptor/scaffold proteins that dynamically assemble a wide range of complexes in response to stimulation of most 7 TMRs [171,179]. The hypothesis is that overexpression of arrestins can either enhance homologous GPCR desensitisation or enhance arrestin-dependent signalling. To determine if CXCR4-mediated calcium

release is arrestin-dependent, MCF-7 cells were transfected (refer to section 2.7.1.; page 56) with pArr2.EGFP, pArr3.EGFP and pA2-Mut.EGFP plasmids DNA. Control cells were transfected with pEGFP.C2 plasmids, which leads to an expression of green fluorescent protein but no signalling function.

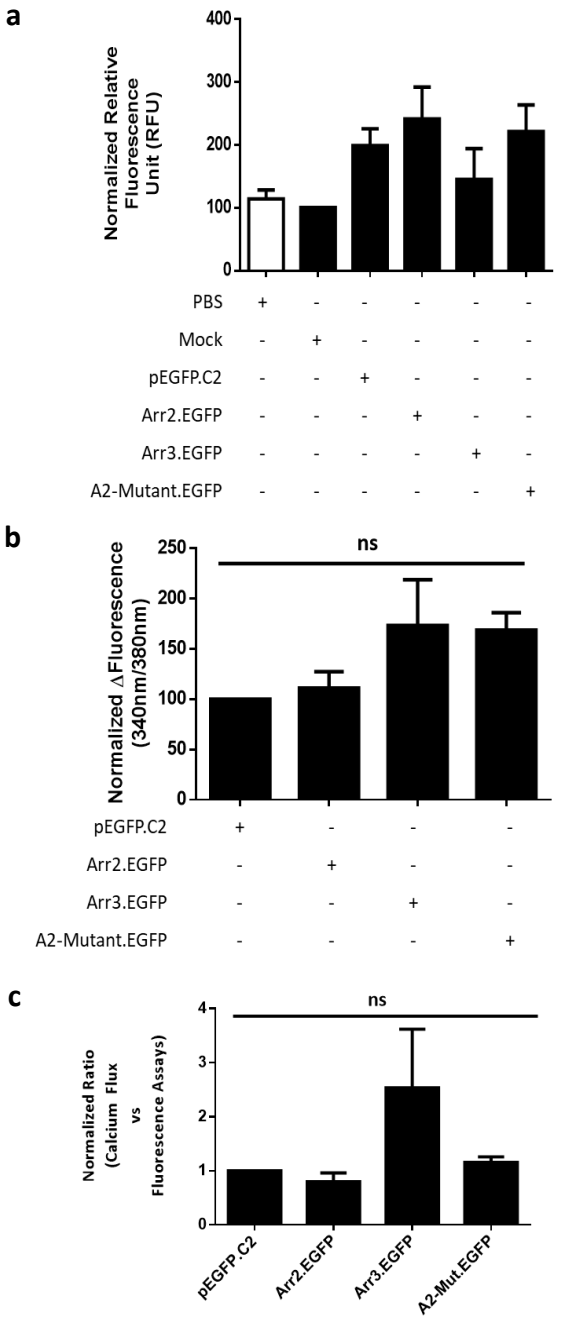


Figure 5.3: pArr3.EGFP and pA2-Mut.EGFP transfected MCF-7 cells seem to slightly increase calcium release. (a) Fluorescence assays using 2 µg plasmids DNA coding for different EGFP tagged arrestin subtypes compared to mock transfected MCF-7 cells. (b) Calcium release of MCF-7 cells transfected with 2 µg plasmid DNA coding for different EGFP tagged arrestin subtypes upon 10 nM CXCL12 activation with pEGFP.C2 as control. (c) Ratio of calcium flux of transfected MCF-7 cells vs expression levels of arrestins in transfected MCF-7 cells. Results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Dunnett's multiple comparison, ns = not significant, $p>0.05$).

Figure 5.3a shows successful transient transfection of different EGFP tagged arrestins subtypes in MCF-7 cells using fluorescence assays. In figure 5.3b, MCF-7 cells that have been transiently transfected with pArr3.EGFP and pA2-Mut.EGFP plasmids showed a trend of increase, though not significant, in calcium release upon stimulation by 10 nM CXCL12 compared to control pEGFP.C2 transfected MCF-7 cells. An increase in calcium signalling when there was an overexpression of arrestin 3 and mutated arrestin 2 suggest that CXCR4-mediated calcium release in MCF-7 cells may not have occurred via 'classical' G-protein signalling, in which arrestin acts as a desensitising protein.

Quantification by ratio in relative fluorescence unit (figure 5.3c) accounts for the difference in calcium release of transfected MCF-7 cells to expression levels of different arrestins' subtypes and the results showed that there was a trend towards an increase in calcium release comparative to an increase in the level of arrestin 3 expression. Though the results were not significant, they suggest that arrestin 3 play a role in calcium release via CXCR4 upon CXCL12 activation, but not arrestin 2 as pArr2.EGFP and pA2-Mut.EGFP overexpressed cells didn't show any difference in calcium release. Analysis of the data suggested that arrestins not only mediate desensitisation of G-protein signalling, but may also act as signal transducers themselves [109].

5.3.2. Arrestin 3 overexpression slightly increase MCF-7 cells migration in wound healing assays via CXCR4 receptor upon CXCL12 activation

Classically, arrestins terminate receptor signalling. After receptor activation, arrestins desensitise phosphorylated GPCRs, blocking further activation and initiating receptor internalisation. Arrestins are required for the desensitisation of several chemokine receptors, including CCR2, CXCR1, CXCR2, CCR5 and chemokine receptor of interest, CXCR4, which is primarily activated by CXCL12 [14,81,246,284,368,369]. It has been shown that arrestins are required in CXCR4-mediated lymphocyte chemotaxis [182] but the precise role of arrestins in leukocyte chemotaxis has not been fully defined. Aberrant expression of CXCR4 in human breast cancer correlates with the metastasis to tissues secreting CXCL12, and recently arrestins have emerged as key regulators of migration of cells. However, the involvement and role of arrestins in migration of these cancer cells has not yet been established. Here, we investigated the role of arrestins in

by chemically transfecting MCF-7 cells and using wound healing assays to assess migration of cells as shown in figures 5.4 and 5.5.

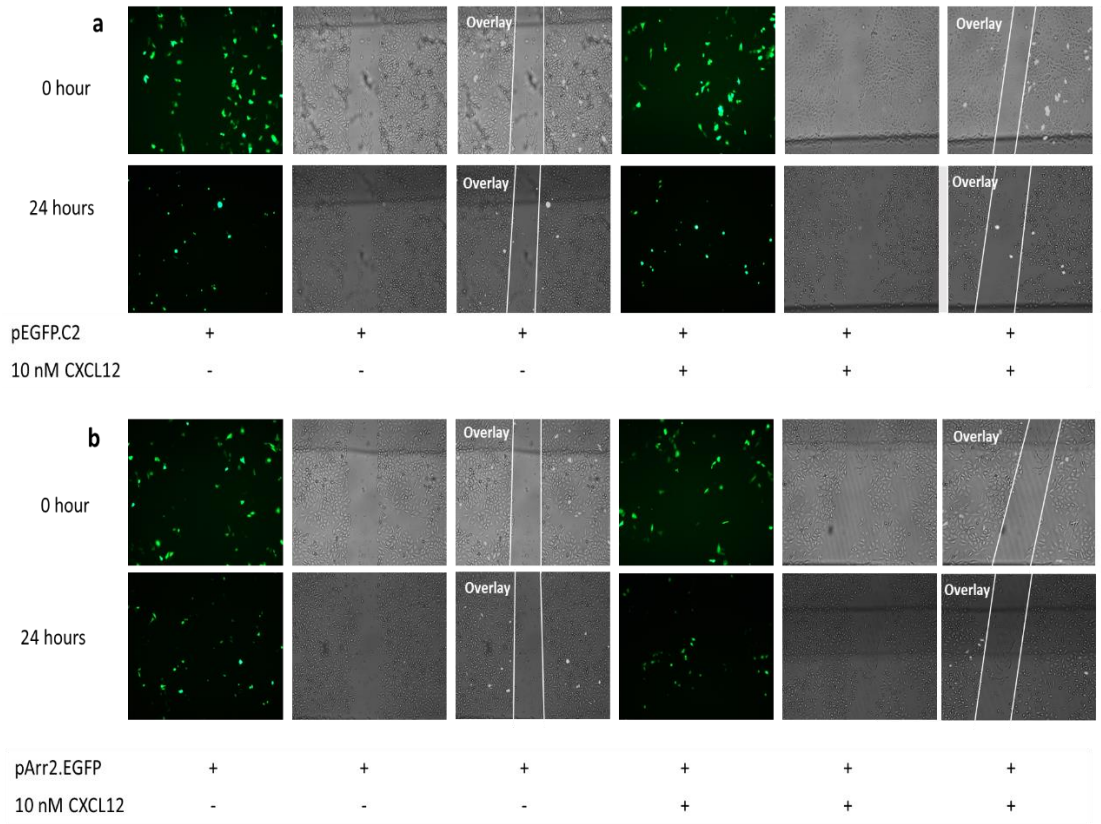


Figure 5.4: Wound healing assay of chemically transfected MCF-7 cells. MCF-7 cells were transfected with 2 µg plasmids DNA coding (a) non-functional pEGFP.C2 as a control and (b) pArr2.EGFP for 24 hours, overexpressing arrestin 2. After 24 hours of transfection, cell migration was induced with 10 nM CXCL12 and measured after 24 hours. Acquired with Leica imaging suite with 10X magnification.

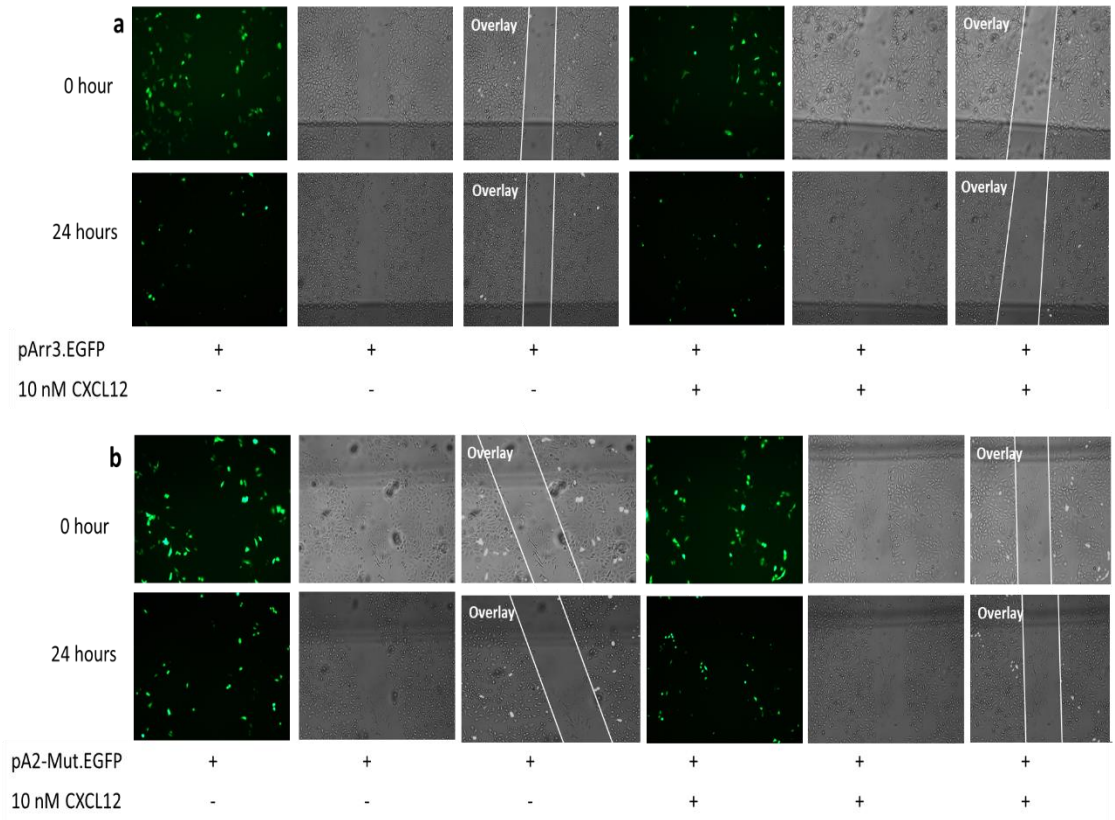


Figure 5.5: Wound healing assay of chemically transfected MCF-7 cells. MCF-7 cells were transfected with 2 µg plasmids DNA coding (a) pArr3.EGFP and (b) pA2-Mut.EGFP for 24 hours, overexpressing arrestin 3 and mutated arrestin 2. After 24 hours of transfection, cell migration was induced with 10 nM CXCL12 and measured after 24 hours. Acquired with Leica imaging suite with 10X magnification.

Pre-treatment for 24 hours with 10 nM CXCL12, pArr3.EGFP MCF-7 cells show a trend of increase of migration of cells into the wound (figure 5.5). Arrestin 3 overexpression, though not significant, appeared to increase MCF-7 migration slightly. This further indicate that both calcium release and migration of MCF-7 cells does not occur via 'classical' G-protein signalling as there was an increase in calcium release and migration of MCF-7 cells overexpressing arrestin 3, suggesting that arrestin 3 plays a role in CXCR4/CXCL12 signalling pathway of MCF-7 breast cancer cells.

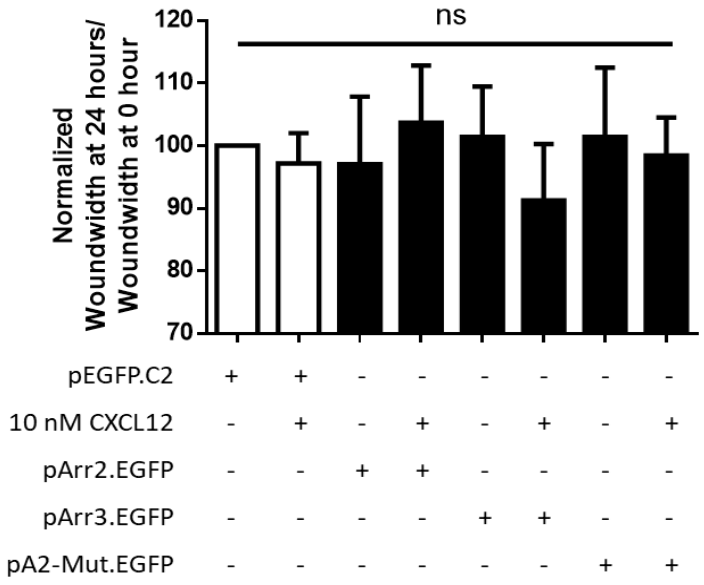


Figure 5.6: Quantification of migration of chemically transfected MCF-7 cells into the wound in the presence or absence of CXCL12. A value of 1 denotes no migration occurred whereas a value < 1 denotes cell migration. MCF-7 cells were chemically transfected for 24 hours using 2 µg plasmids DNA coding for pArr2.EGFP, pArr3.EGFP and pA2-Mut.EGFPand also pEGFP.C2 as a control. Pre-treatment with 10 nM CXCL12 for 24 hours before data analysis. All results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, ns = not significant, p>0.05).

Figure 5.6 show representative results from chemically transfected MCF-7 cells in wound healing assays; pArr3.EGFP transfected MCF-7 cells' migration show a small, but not significant, trend of increase compared to control pEGFP.C2 cells, but not pArr2.EGFP and pA2-Mut.EGFP transfected cells. This implies that arrestin 3 is the important arrestin subtype in CXCR4/CXCLl2 signalling axis in MCF-7 cells. However, it is difficult to say with certainty that the role of arrestins in this assay when the positive control was not robust, which may be due to loss of cells at the end of the experiment. Since the transfection efficiency was low and variable, transient transfected MCF-7 cells do not provide a reliable test system. As mentioned previously, we also observed a loss of cells during the timeframe of the experiment (figures 5.4 and 5.5), which predominantly seemed to affect the transfected cells. The reason for this could be that after 72 hours of incubation, these cells are more mobile after transfection and therefore more likely to come off the plastic well. However, it confirms that this method is not reliable enough to understand the function of arrestins in cell migration.

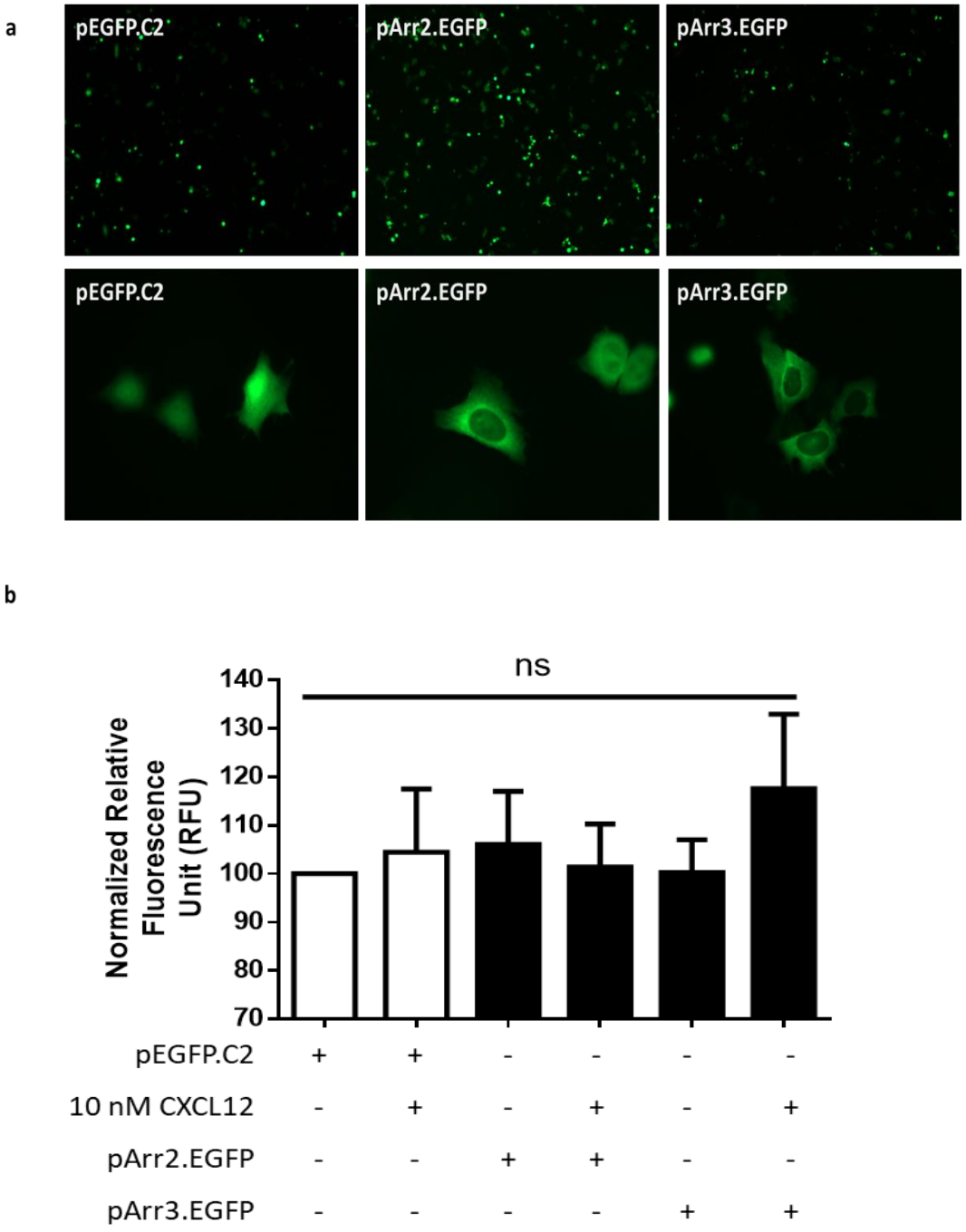


Figure 5.7: Quantification of migration of chemically transfected MCF-7 cells using Boyden Chamber migration assay and fluorescence was measured after 24 hours in the presence or absence of CXCL12. MCF-7 cells were chemically transfected for 24 hours using 2 µg plasmid DNA coding for pArr2.EGFP, pArr3.EGFP and pEGFP.C2 as a control (a) Micrographs of transfected MCF-7 monolayers with pArr2.EGFP, pArr3.EGFP, and pEGFP.C2 as control. Images representative of population. Acquired with Leica imaging suite with 10x and 63X magnifications. (b) Relative fluorescence unit measured after pre-treatment with 10 nM CXCL12 after 24 hours. All results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, ns = not significant, $p>0.05$).

As described previously, there was high variability and inconsistency in the wound healing assays, hence alternative cell migration assays can be considered. The most widely accepted cell migration technique is the Boyden chamber assay. We therefore used this experimental set-up on the transiently transfected MCF-7 cells. The chamber was suspended over a larger well which contain medium as control and 10 nM CXCL12 as the chemotaxis-inducing agent (refer to section 2.9.2.; page 58).

Fluorescence images shown in figure 5.7a demonstrated that there was effective chemical transfection of MCF-7 cells, but looking at figure 5.7b, there was a variability between different experiments. However, even with these variations in experiments, a trend of an increase in migration of transfected pArr3.EGFP cells compared to pArr2.EGFP transfected cells was observed, which agreed with data shown in figure 5.6. This data further implies that arrestin 3 is the important arrestin subtype that plays a role in CXCR4/CXCL12 pathway in migration of MCF-7 cells. This trend, however, was not significantly different to the control.

There is limited literature that supports efficiency of chemical transfection of MCF-7 cells which indicates that MCF-7 cells may be difficult to be transfected effectively. Overall, the MCF-7 cells did not migrate very well at all under these conditions and are therefore, not a good model system to investigate the influence of arrestin on migration.

5.3.3. Overexpression of arrestin has no obvious disruptive effect on MCF-7 cells' actin polymerisation upon CXCL12 activation

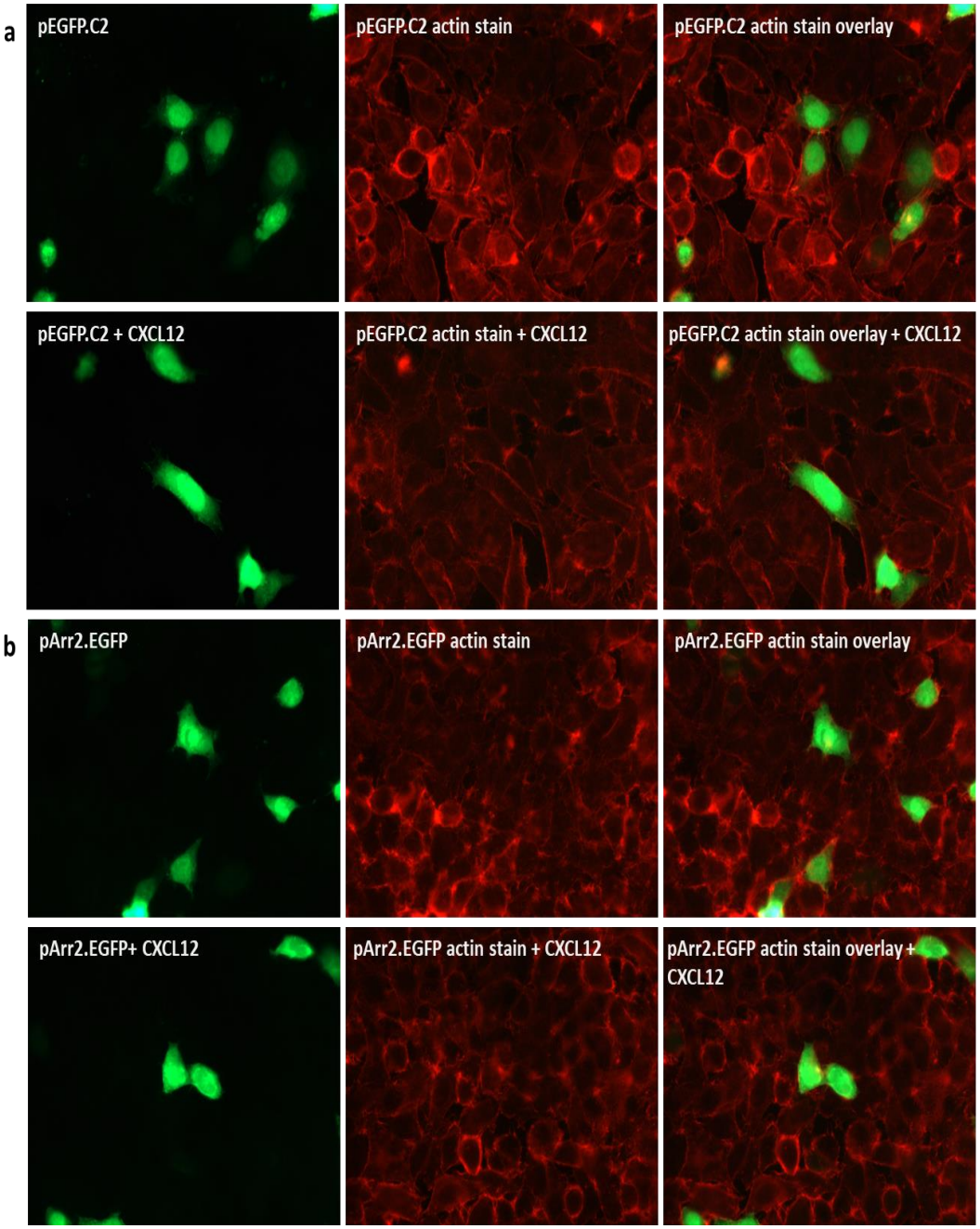


Figure 5.8: Arrestin 2 overexpression has no obvious effect on actin polymerisation in MCF-7 cells. Micrographs of MCF-7 monolayers chemically transfected for 24 hours using 2 µg plasmids DNA coding for pArr2.EGFP (green) and pEGFP.C2 (green) as a control and then treated with 10 nM CXCL12 for 15 minutes. Cells were fixed with 4% paraformaldehyde and stained with Phalloidin CruzFluor 594 (red). Images representative of population. Acquired with Leica imaging suite using 10X magnification.

Actin filaments staining with fluorescent phalloidin is a useful imaging tool to investigate the distribution of F-actin in cells. Here, we investigated the effects of arrestin on actin cytoskeleton of MCF-7 cells. Phalloidin CruzFluor 594 conjugate is a deep red fluorescent conjugate that binds to actin filaments, stabilising actin filaments through the prevention of filament depolarisation. Actin is used by the cell for mechanical processes such as growth, scaffolding of the cytoskeleton, and movement. Therefore, there is importance in investigating actin as disruption of the actin equilibrium destroys cellular functioning and blocks migration [370,371].

Fluorescence microscopy was used to determine if actin cytoskeleton was disrupted by overexpression of arrestins 2 and 3. By transfecting MCF-7 cells with pArr2.EGFP and pArr3.EGFP plasmids compared to control pEGFP.C2 MCF-7 cells and staining the cells with Phalloidin Cruzfluor 594 (red fluorescent conjugate), we could see if changes in levels of arrestins expression would have an obvious effect on actin cytoskeleton upon CXCL12 activation. These cells were grown as a monolayer on coverslips to high confluence (refer to section 2.10.1.; page 60) and chemically transfected for 24 hours and then were stimulated with 10 nM CXCL12 for 1 hour prior to fixing with 4% paraformaldehyde and staining.

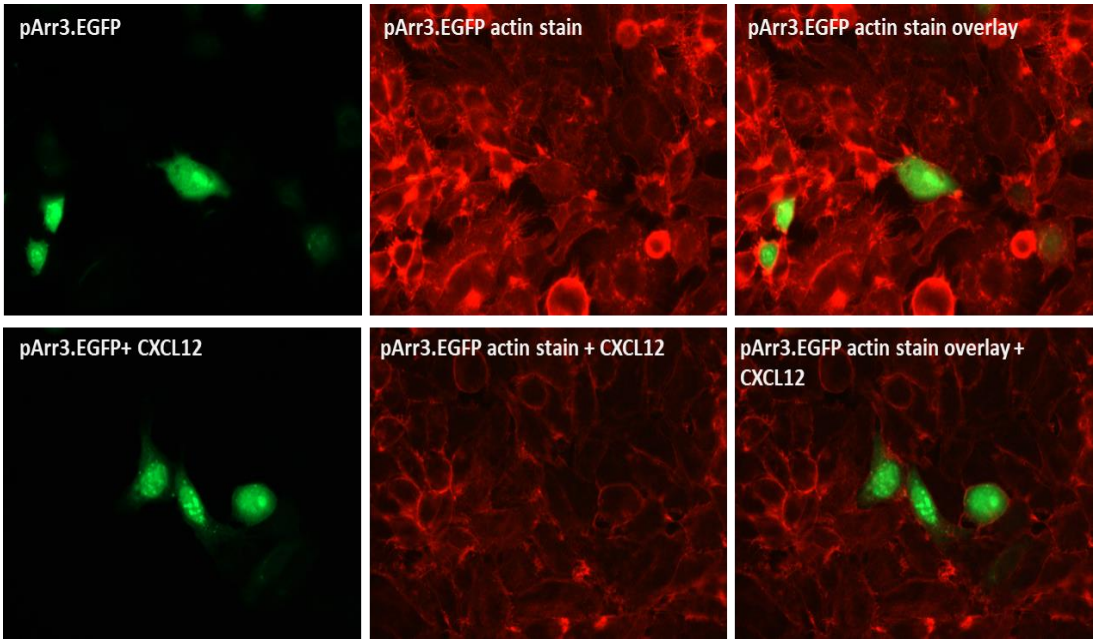


Figure 5.9: Arrestin 3 overexpression has no obvious effect on actin polymerisation in MCF-7 cells. Micrographs of MCF-7 monolayers chemically transfected for 24 hours using 2 µg plasmids DNA coding for pArr3.EGFP (green) and then treated with 10 nM CXCL12 for 15 minutes. Cells were fixed with 4% paraformaldehyde and stained with Phalloidin CruzFluor 594 (red). Images representative of population. Acquired with Leica imaging suite using 10X magnification.

Arrestin 2 and 3 overexpression did not have an obvious effect on actin polymerisation in MCF-7 cells (figures 5.8, 5.9). Although actin stress fibres were not widely evident due to the low magnification of images and the small sized MCF-7 cells, the distribution of actin was uniform. There was not any obvious disruption to actin localisation and uniformity of actin.

MCF-7 cells are rather small in size; hence it is very difficult to identify cellular structures. MCF-7 cells also tend to grow on top of each other, instead of forming a uniform monolayer, so it can be difficult to observe and identify cellular structures clearly. MCF-7 cells have been proven to have high level of CXCR4 expression, but the inability to clearly observe actin stress fibres across the cells was disappointing. The majority of actin fibres are situated along the plasma membrane surrounding the cells and neither stimulation by CXCL12 nor overexpression of arrestins showed an obvious change in the localisation of the actin fibres.

5.3.4. Investigation of the role of arrestins in leukemic T lymphocytes, Jurkat cells

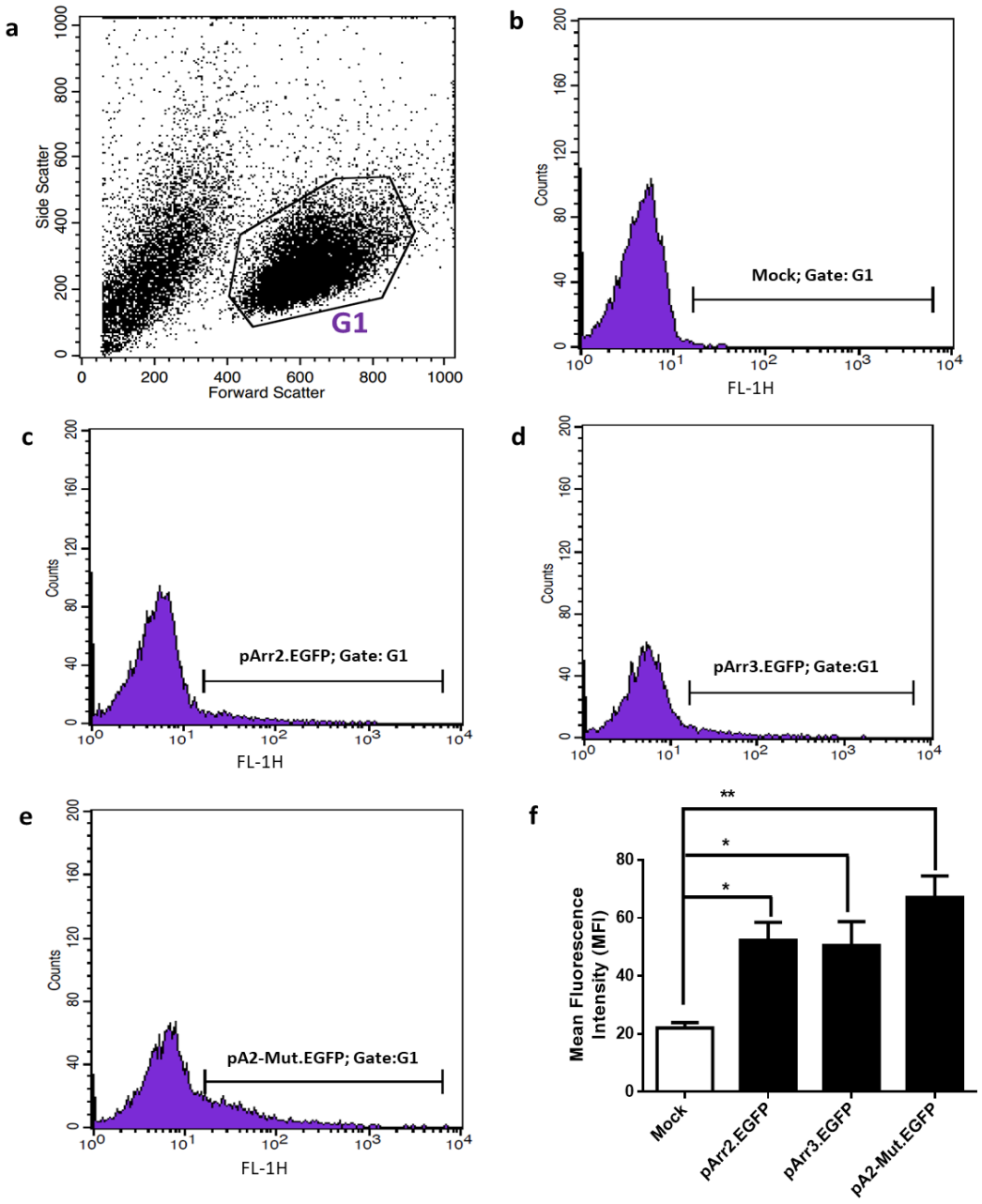


Figure 5.10: Flow cytometry. (a) G1 gated population was used, to show transient transfection of Jurkat cells by electroporation transfection in the presence or (b) absence (mock) of DNA plasmids coding (c) pArr2.EGFP, (d) pArr3.EGFP and (e) pA2-Mutant.EGFP. (f) Quantification of mean of fluorescence intensity based on gated population of Jurkat cells, G1. Results represent the mean ± SEM of 3 independent experiments. (One-way ANOVA, Dunnett's multiple comparison, * = p≤0.05, ** = p≤0.01)

Since non-invasive adherent breast cancer cell lines were difficult cell lines to work with and had low transfection efficiency, Jurkat cells (leukemic T-lymphocytes) were

an alternative cell line to work with to investigate CXCR4/CXCL12 signalling axis. As discussed previously, Jurkat cells have been proven to express high levels of CXCR4, and also have been widely studied in the group as a model system to look at CXCR4 function [286]. There is literature suggesting that cellular background can be important for distinct signalling pathways [286] used by the CXCR4 receptor, hence it is important to establish the roles of arrestins in a different cell line, and to use another cell line to define CXCR4/CXCL12 signalling axis.

Transfection of these suspension cells was different from adherent breast cancer cells, since Jurkat cells were not transfected with a chemical agent but by using electroporation transfection, Amaxa Nucleofector programme X-005. Nucleofection is an electroporation-based transfection method which enables transfer of nucleic acids such as DNA or RNA into cells by applying a specific voltage and reagents. Jurkat cells were transfected with pArr2.EGFP, pArr3.EGFP and pA2-Mut.EGFP and also pEGFP.C2 as non-functional control (refer to section 2.6.1.; page 55), using appropriate Nucleofector programme X-005 for high expression level. This particular programme has 84%±3 efficiency rate and 75%±11 viability with 2 µg DNA and 1 X 10^6 cell count according to literature and optimised protocol provided by Amaxa, Lonza [372,373]. With Nucleofection, even hard-to-transfect cell lines can be transfected with high efficiency, which would allow us to study the role of arrestins in CXCR4/CXCL12 signalling in Jurkat cells more in-depth.

Jurkat cells were transfected for 24 hours and activated with 15 nM CXCL12 for 1 hour. Arrestin transfected cells showed a significant increase in fluorescent intensity compared to mock transfected control cells as determined by flow cytometry (figure 5.10f) using the forward/side scattered plot, marked by G1 population (figure 5.10a). Flow cytometry is a useful tool to analyse transfection efficiency in electroporated transfected Jurkat cells as it allows rapid, accurate and simple of collection of data related to many parameters from a heterogenous fluid mixture containing live cells. Figures 5.10b – 5.10e showed that there was a difference between mock transfected and arrestins transfected cells and since the mean fluorescence values obtained in mock transfected cells was significantly lower than in the EGFP-arrestin transfected cells (figure 5.10f), we have shown that there was effective transient transfection of Jurkat cells. However, there was a lower transfection efficiency rate than what is stated in

literature, concluding that Jurkat cells are not easily transfected. As enough viable cells were still available, further experiments to determine the role of arrestins in Jurkat cell line in CXCL12-induced migration via CXCR4 receptor were undertaken.

Figure 5.11 shows flow cytometry of CXCR4 internalisation after electroporation transfection of Jurkat cells with different EGFP tagged arrestin subtypes and induction with 15 nM CXCL12.

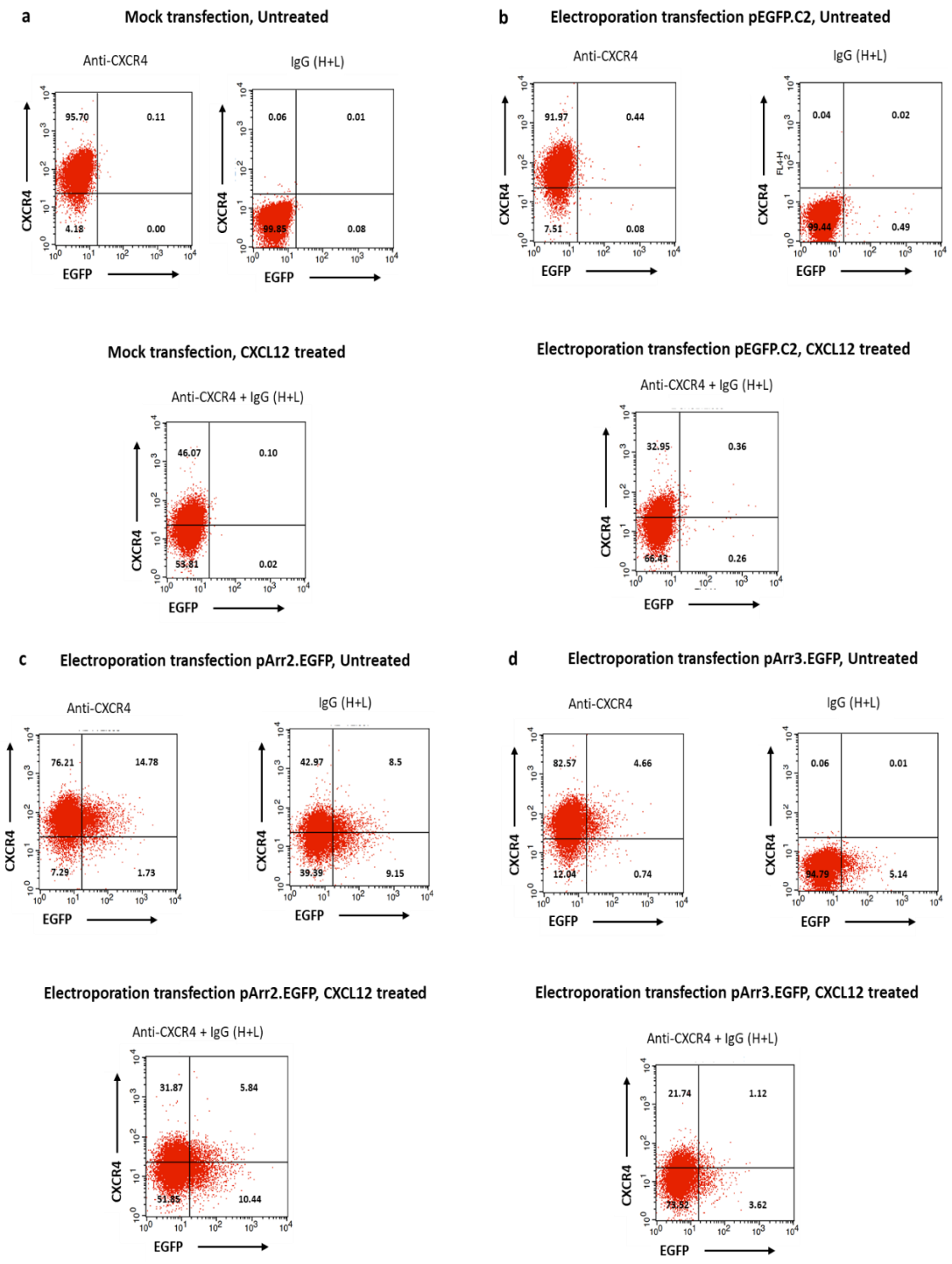


Figure 5.11: Flow cytometry of arrestin transfected Jurkat cells. Flow cytometry analysis was carried out following electroporation transfection of Jurkat cells in the presence or (a) absence (mock) of DNA plasmids coding (b) pEGFP.C2 as non-functional control, (c) pArr2.EGFP and (d) pArr3.EGFP. Electroporation transfection was conducted 24 hours before the induction of CXCR4 internalisation with 15 nM CXCL12. The percentage of cells is shown in each quadrant.

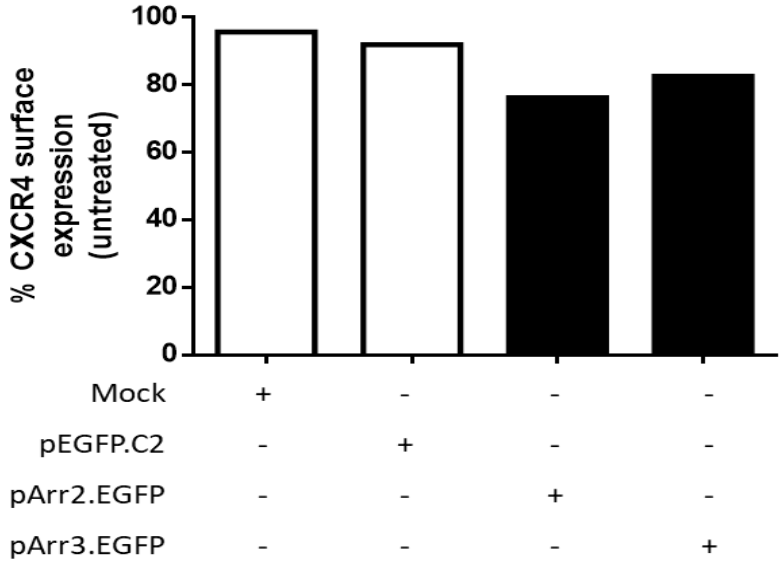


Figure 5.12: Comparison of percentage CXCR4 expression in the presence or absence (mock) of DNA plasmids coding pArr2.EGFP and pArr3.EGFP and also pEGFP.C2 as non-functional control. This data represents percentage of CXCR4 surface expression in transfected cells in the absence of CXCL12. Preliminary data represent one experiment.

Figures 5.12 and 5.13 show the comparison of percentage CXCR4 expression in the presence of DNA plasmids coding pArr2.EGFP, pArr3.EGFP and pEGFP.C2 as non-functional control vs mock transfected Jurkat cells. From these data, we can start to investigate whether overexpression of arrestins does affect the expression of CXCR4 on the cell surface. Preliminary data shows that overexpression of arrestins did not affect CXCR4 cell surface expression in Jurkat cells.

The relative CXCR4 surface expression was calculated as a percentage using the following: 100 x (mean channel of fluorescence [stimulated])/ (mean channel of fluorescence [medium]). The preliminary experiment of staining CXCR4 in arrestin transfected cells on ice (in either the presence or absence of 15 nM CXCL12) has shown that there was no difference in internalisation rate between mock transfected cells and arrestin transfected cells upon CXCL12 stimulation.

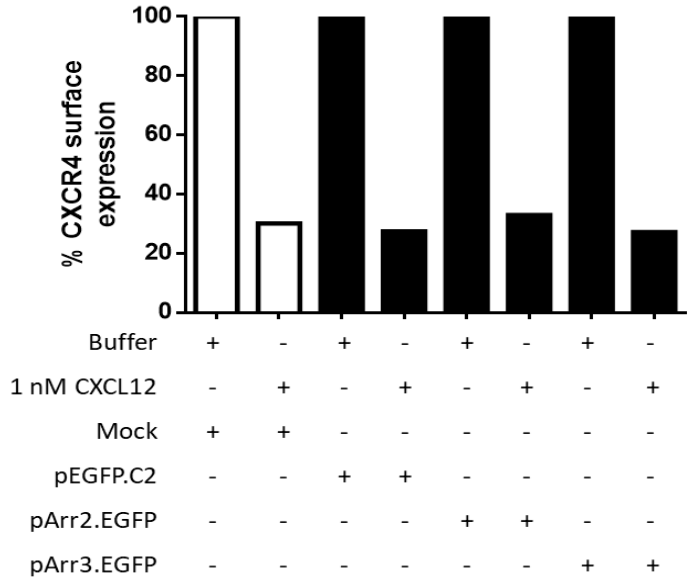


Figure 5.13: Preliminary data showed that arrestins may not be required for CXCL12-induced internalisation of CXCR4. CXCL12-induced internalisation of CXCR4 in Jurkat cells as deduced by flow cytometry following electroporation transfection in the presence or absence (mock) of 2 µg plasmids DNA coding pEGFP.C2 as non-functional control, pArr2.EGFP and pArr3.EGFP to overexpress arrestins 2 and 3. The relative CXCR4 surface expression of transiently transfected Jurkat cells following incubation with 15 nM CXCL12 or buffer alone. Preliminary data represent one experiment.

In the 'classical' model of GPCR regulation when receptor is activated and phosphorylated, CXCR4 signalling is rapidly desensitised after ligand binding by receptor internalisation. This is achieved by the binding of arrestins, which sterically block further interactions between GPCR and G-proteins , which would then mediate receptor internalisation through clathrin coated pits [2,69,142].

Transfected Jurkat cells were activated with 15 nM CXCL12 for 1 hour before treatment with anti-CXCR4 primary antibody for 1 hour and then stained with anti-mouse IgG (H+L) 647 secondary antibody for 1 hour before analysing in flow cytometry (refer to section 2.11.; page 62). The preliminary data may suggest that internalisation of CXCR4 is not arrestin-dependent as there was not any increase of internalisation rate in overexpression of arrestins. However, since the data was based on one experiment with a high internalisation rate in mock transfected, any further conclusion should be avoided. It is difficult to observe differences in internalisation rate from these experiments when there could be other factors affecting internalisation rates, such as the carboxyl-terminus of the receptor, the type of ligand, the cell type and the

phosphorylation status of CXCR4 receptor in cells [313]. Therefore, exploring other different methods to show the effects of arrestin overexpression on CXCR4 receptor in Jurkat cells via CXCL12 signalling transduction is important.

5.3.5. Investigation of overexpression of arrestins in migration of leukemic T lymphocytes, Jurkat cells via CXCR4 upon CXCL12 activation

Arrestins are required for the desensitisation of several chemokine receptors, including CXCR4, and so there is importance in studying the role of arrestins and how they affect CXCL12-induced migration via CXCR4 – through receptor desensitisation or localised scaffolding of cytoskeletal signalling molecules [14,284]. Furthermore, the mechanism by which arrestins mediate migration may differ with cell and receptor type [81].

In addition to regulating receptor stimulated G-protein signalling, arrestins are also capable of initiating distinct signalling patterns. These patterns are often both spatially and temporally distinct from G-protein mediated signalling which result in cellular consequences, including migration of cells [108,193].

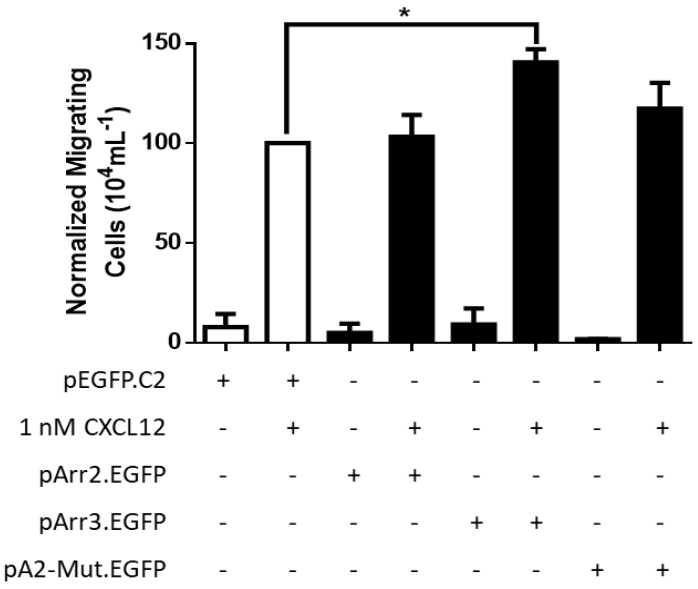


Figure 5.14: Arrestin 3 overexpression significantly increase migration of Jurkat cells via CXCR4 upon CXCL12 activation. Jurkat cells were transiently transfected by electroporation transfection with 2 µg plasmids DNA coding pEGFP.C2 as non-functional control, pArr2.EGFP, pArr3.EGFP and pA2-Mutant.EGFPto overexpress arrestin 2, 3 and mutated arrestin 2. Cells were transfected for 24 hours. Cell migration towards 1 nM CXCL12 in pEGFP.C2 control cells or overexpressed arrestin 2, 3 and mutated arrestin 2 Jurkat cells. All results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * = p≤0.05).

Transfected Jurkat cells were incubated for 5 hours with 1 nM CXCL12 in chemotaxis assays before cells were counted and data analysed (refer to section 2.9.1.; page 58). We observed a significant increase in migration of cells that was transfected with plasmids DNA coding pArr3.EGFP (figure 5.14) in CXCR4 signalling axis upon 1 nM CXCL12 activation. This indicated that arrestin 3 may play a role in migration of Jurkat cells, but not arrestin 2 subytype as there was not much difference in migration between control pEGFP.C2, pArr2.EGFP and pA2-Mut.EGFP cells. These results indicate that arrestin 3, and not arrestin 2, is required in migration of cells is supported by studies performed by Fong *et al*. (2002) where they have shown that CXCR4-mediated lymphocyte chemotaxis was defective in arrestin 3 and G-protein coupled receptor kinase 6 (GRK6)- knockout mice [145]. Investigations of knockdown arrestins in Jurkat cells using small interfering RNA (siRNA) was then undertaken to confirm the role of arrestins in CXCL12-induced migration in Jurkat cells.

siRNA was electroporation transfected to knockdown arrestins in Jurkat cells. siRNA-mediated gene knockdown (Dharmacon, USA) is a powerful tool that has been used to identify gene function and to map out biological pathways. Scrambled siRNA is used as a negative control siRNA, as they are designed to have no known target in the cells and are important in distinguishing sequence-specific silencing from non-specific effects in the RNAi experiment. Jurkat cells were electroporated transfected with siRNAs (refer to section 2.6.2.; page 55) for 48 hours before stimulation with 1 nM CXCL12 in chemotaxis assays for 5 hours. Electroporation transfection was the method chosen for siRNA delivery because suspension cells are not compatible with lipid-mediated transfection.

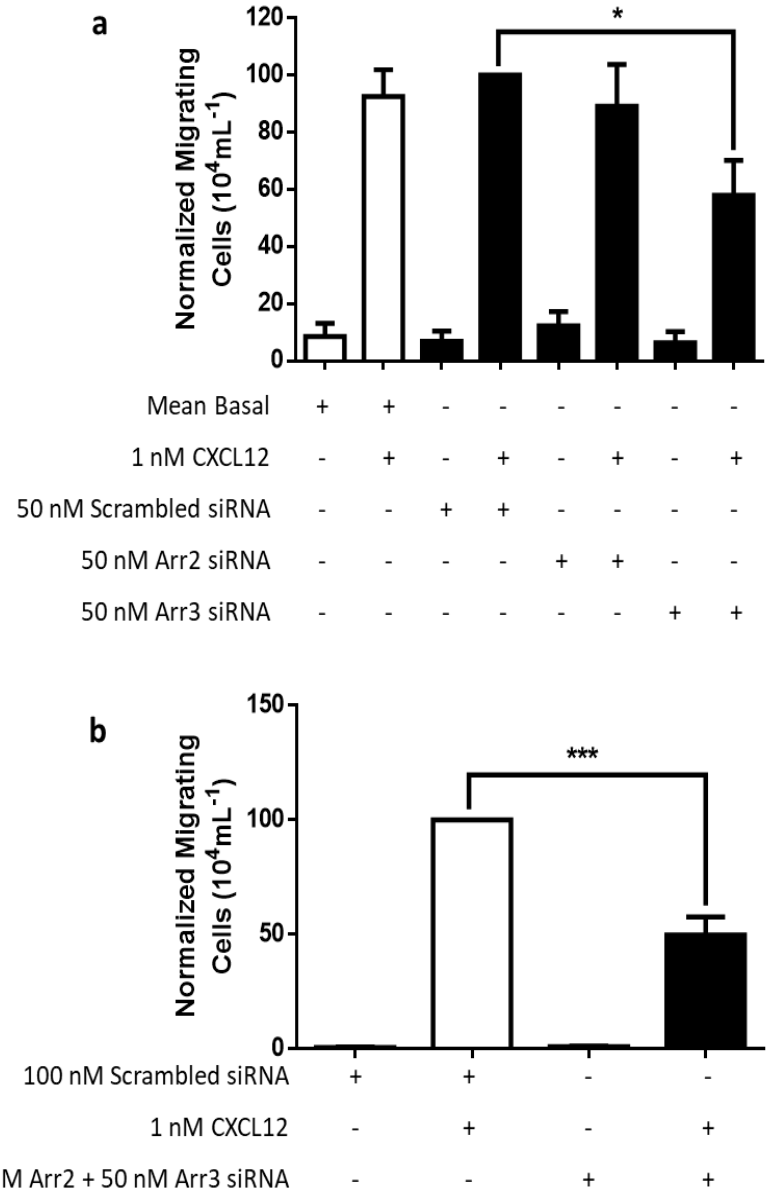


Figure 5.15: Arrestin 3 plays a role in migration of Jurkat cells via CXCR4 upon CXCL12 activation. Jurkat cells were transiently transfected by electroporation transfection with (a) 50 nM Arrestin 2 siRNA (Arr2 siRNA), Arrestin 3 siRNA (Arr3 siRNA) and 50 nM scrambled siRNA as non-functional control to knockdown arrestins 2 and 3 in Jurkat cells (b) 100 nM scrambled siRNA and combination of 50 nM Arr2 and Arr3 siRNAs. Cell migration towards 1 nM CXCL12 in scrambled siRNA control cells or knockdown arrestins 2 and 3 in Jurkat cells. All results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * = $p \leq 0.05$, *** = $p \leq 0.001$)

In Jurkat cells with knockdown arrestin 3, there was a significant reduction in migrating cells compared to negative scrambled siRNA (figure 5.15a). This data confirmed that CXCL12-induced migration might be arrestin 3-dependent signalling. Another experiment was done to see if there was a cumulative effect when both arrestin 2 and 3 were knocked down with electroporation transfection and incubated for 48 hours before analysis (figure 5.15b). Results showed that upon CXCL12 activation in chemotaxis assay for 5 hours, half of the migratory cells were significantly reduced, which was very similar to the data where only arrestin 3 was knocked down. There was no indication of cumulative effect from combination knockdown of arrestins 2 and 3. Hence, these data imply that the reduction in migration of cells is due to arrestin 3 subtype and that CXCL12-induced migration via CXCR4 in Jurkat cells is arrestin 3-dependent signalling.

siRNA knockdown technique is useful to assess the function of a protein within cells as they are involved in the RNA interference (RNAi) pathway. To assess the results from the knockdown experiments, we used the western blot technique (refer to section 2.12.; page 63), which usually indicates the presence or absence of proteins. Different antibodies with different epitope specificities (table 5.1) were used to observe if there was a knockdown of arrestins in samples.

Table 5.1: Different primary antibodies from Santa Cruz Biotechnology

Antibodies used for arrestin 2/3 Western Blot	**Source**
β-Arrestin-1/2 Antibody (H-290): sc-28869	• Rabbit polyclonal IgG • Epitope corresponding to amino acids 7-290 mapping at the N-terminus of β-arrestin-1 (arrestin 2) of human origin
β-Arrestin-1/2 Antibody (21-B1): sc-53781	• Mouse monoclonal IgG_1 • Raised against β-arrestin-1 (arrestin 2) of human origin
β-Arrestin-1/2 (A-1): sc-74591	• Mouse monoclonal IgG_1 (kappa light chain) • Raised against amino acids 7-290 mapping at the N-terminus of β-arrestin-1 (arrestin 2) of human origin

Unfortunately, we did not manage to observe any bands, even in the untreated control Jurkat cells where arrestin band should be visible at molecular weight of 55 kDa. We have done numerous changes to the method and followed different optimised protocols, including changing the primary antibodies, but only the western blot loading control, beta actin, was visible on the blot, whereas arrestin was never detected reliably (figure 5.16). One reason for this is that the antibodies commercially available for detecting arrestins are not very reliable. Another approach that could be used is real-time PCR (RT-PCR) to check for the presence of the mRNA, which is a very sensitive method, but doesn't give an accurate prediction of expected protein levels. Unfortunately, we were not able to use the RT-PCR approach in the preparation of this thesis.

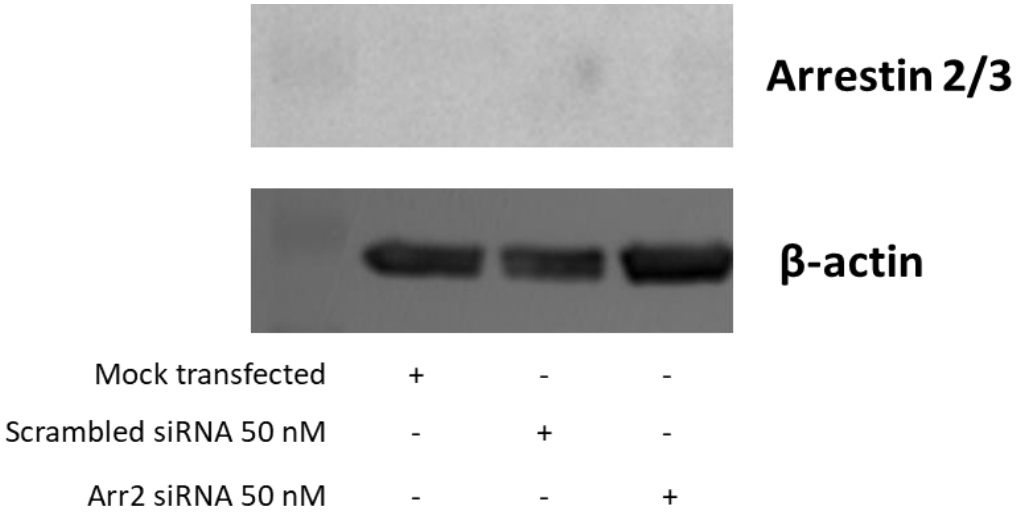


Figure 5.16: Western blot analysis of arrestin expression in Jurkat cells after knockdown, where β-actin acts as loading control. Primary antibody, β-Arrestin-1/2 (21-B1) mouse monoclonal (1:500 dilution) and secondary antibody anti-mouse pox (1:5000 dilution) were used in arrestin2/3 western blot.

It is disappointing that we could not show that there definitely was a knockdown of arrestins triggering mRNA degradation and suppression of gene expression for arrestin which contributed to the reduction of migratory cells. However, by combining the data from overexpression and knockdown of arrestins in Jurkat cells, they are consistent in suggesting that arrestin 3 plays a role in CXCL12-induced migration via CXCR4 in Jurkat cells.

5.3.5. Investigation of overexpression of arrestin 3 in MAPK and PKC signalling cascades upon CXCL12 activation via CXCR4 receptor in Jurkat cells

PKC family members regulate numerous cellular responses including gene expression, protein secretion, cell proliferation, and the inflammatory response [126]. When cells are stimulated, PKC is transiently activated by diacylglycerol which is produced in the membrane during signal-induced turnover of inositol phospholipids [130]. Activation of chemokine receptors can lead to phosphorylation of the receptor via GRK and PKC [141,142,343]. Classically, G-protein stimulation via second messenger effectors like PKC and protein kinase A (PKA) promotes ERK1/2 phosphorylation and nuclear translocation for modulating transcription [374].

Recently, it has become evident that arrestins serve as molecular scaffolds for numerous signalling proteins [80], but the consequences of these signalling events are still poorly understood. Numerous receptors which require arrestins for migration also promote arrestins' dependent association with MAPKs [169,170,180,182,375]. It is important to study the MAPK pathway and to understand its downstream signalling cascades as many extracellular growth factors and stresses converge on MAPKs for regulating cellular processes, such as cell proliferation, survival and differentiation [171].

The ERK MAPK signalling pathway is the model for arrestin mediated signalling, whereby GPCRs activate MAPK through the G-protein subunits upon agonist stimulation [376,377]. Arrestins scaffold the MAP kinase signalling molecules, including MAP kinase (MEK1) and MAP kinase (ERK), leading to phosphorylation and activation of ERK1/2 [179]. An association with arrestin-dependent ERK activation has been described in previous researches but how arrestins affect these downstream signalling effectors is still not fully understood [179].

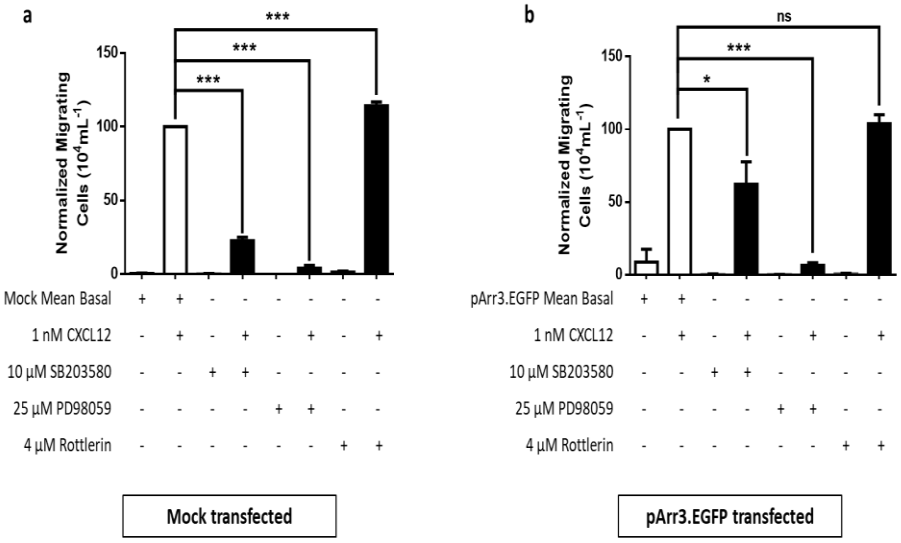


Figure 5.17: Effects of overexpression of arrestin 3 in chemotaxis assays on PKC and MAPK signalling pathways. Jurkat were transiently transfected by electroporation transfection, in the (a) absence (mock) or presence of 2 µg plasmids DNA coding (b) pArr3.EGFP to overexpress arrestin 3. Cells were incubated in the absence and presence of 10 µM SB203580 (selective p38 MAPK inhibitor), 25 µM (MEK1/2 inhibitor) and 4 µM Rottlerin (non-selective PKC inhibitor) for 30 minutes and cell migration towards 1 nM CXCL12 in overexpressed arrestin 3 Jurkat cells vs control mock transfected Jurkat cells analysed after 5 hours. All results represent the ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * = p≤0.05, *** = p≤0.001, ns = not significant).

Since we have shown that arrestin 3 plays a role in CXCL12-induced migration via CXCR4 receptor activation in Jurkat cells, it would be worthwhile to investigate the effects of overexpression of arrestin 3 in MAPK and PKC signalling pathways as the next step. Jurkat cells were transfected with DNA plasmids coding pArr3.EGFP for 24 hours and treated with inhibitors for 30 minutes before stimulated in chemotaxis assays for 5 hours (refer to section 2.6.1.; page 55).

ERK1/2 and p38 MAPK are associated with canonical Gβγ signalling to chemotaxis [75] and are also used more generally as markers or receptor mediated protein activation associated with Gβγ and arrestin-dependent signalling. p38 MAPKS are members of the MAPK family which are activated by a variety of environmental stresses and inflammatory cytokines. SB203580 is a selective inhibitor of p38 MAPK. The data showed that in overexpressed arrestin 3 Jurkat cells, there was significantly less inhibition of cells via p38 MAPK pathway when comparing migration of treated pArr3.EGFP cells (figure 5.17b) to treated mock transfected Jurkat cells (figure 5.17a)

with 10μM SB203580. This observation suggests that arrestin 3 plays a role in p38 MAPK signalling cascade that results in migration of cells.

PD98059 is a small molecule inhibitor of MKK/ MEK in MAPK cascade and it acts by binding to the inactivated form of MEK. The results showed that ERK1/2 MAPK pathway may not be arrestin 3-dependent signalling as there was no difference in migration of pArr3.EGFP cells (figure 5.17b) vs mock transfected Jurkat cells (figure 5.17a) when treated with 25μM PD98059. We observed significant inhibition of cell migration towards 1 nM CXCL12 in both conditions.

Figure 5.17 also showed a possible relationship between PKC and arrestin 3 in CXCL12-induced chemotaxis as there was a difference observed between mock transfected and pArr3.EGFP transfected Jurkat cells after PKC inhibition by 4 μM Rottlerin, a non-selective PKC inhibitor. Overexpression of arrestin 3 in Jurkat cells did not significantly increase migration of cells treated with Rottlerin (figure 5.17b), unlike in mock transfected Jurkat cells where PKC inhibition significantly increased migration of cells (figure 5.17a). However, in both conditions, there was no inhibition of migration observed and the difference in numbers of migratory cells between the two conditions were insignificant for us to conclude that there definitely is a link between arrestin 3 and PKC. Since there wasn't any inhibition of migration of cells observed, this further confirmed that CXCL12-induced migration of CXCR4 is not PKC-dependent as discussed in chapter 4.

5.4 Discussion

Canonical signalling by 7TMRs involves ligand binding which induces a change in receptor conformation that causes the activation of heterotrimeric G-proteins, leading to second messengers' generation and downstream signalling. Desensitisation occurs when GRKs phosphorylate the activated receptor and promotes the binding of arrestins, which act sterically to block further G-protein activation [374].

However, in recent years, the roles of arrestins has evolved to become distinct transducers of 7TMR that signals independent of G-proteins and to facilitate the activation of numerous signalling pathways, including MAPKs, c-Src and Akt by acting as multiprotein binding scaffolds [80,109,261]. These diverse downstream signalling cascades involving arrestin mediated signalling results in cellular responses such as chemotaxis [81], cardiomyocyte contractility [378], and the prevention of apoptosis [186]. Chemotaxis mediated by CXCR4 plays a key role in lymphocyte homing and haematopoiesis. Chemokine receptors are GPCRs that mediate chemotaxis in response to inflammatory chemokines released at sites of injury [379].

Arrestins are small family of proteins that are important for regulating signal transduction at GPCRs. Arrestins exist in the cytoplasm in multiple forms; free monomers, free oligomers and microtubule-associated arrestins [380]. Mammalian arrestin family can be broadly classified into visual and non-visual groups [164]. The visual arrestins, arrestin 1 (rod arrestin) and arrestin 4 (cone arrestin) are expressed only in photoreceptors while the non-visual receptors, arrestin 2 and arrestin 3 exist ubiquitously in most cells and interacts with hundreds of GPCRs [164,380]. A prominent difference between arrestin 2 and arrestin 3 is their subcellular localisation [381]. While arrestin 3 appears to be almost exclusively extranuclear, arrestin 2 is found both in the cytoplasm and nucleus [381]. This might clarify why mutated arrestin 2 protein has been observed to be primarily located in the nucleus of the cell (figure 5.2). As discussed previously, there are several recent studies that have found that arrestins mediate various forms of crosstalk between receptor signalling systems due to their position in cells, and this may be due to the fact that most signalling pathways have been found to function as components of larger signalling networks [261].

Arrestins are well known to terminate receptor signalling, and after receptor activation, arrestins desensitise phosphorylated GPCRs, blocking further activation and initiating receptor internalisation [80]. In addition to arrestins' involvement in the internalisation of receptors, arrestins can also function as signal transducers by directly activating pathways such as Akt, MAPK and PI3K, but their role as mediators of receptor desensitisation, scaffolding or as direct signalling modulators to these pathways are still poorly understood [18,81]. Our aim is to study how non-visual arrestins are involved in migration of cells, and also, to see if different cell types have different usage of arrestins leading to migration of cells.

Calcium release upon CXCL12 activation via CXCR4 signalling in MCF-7 cells may not be arrestin-dependent signalling in both 'classical' and 'scaffold' settings, but in migration of MCF-7 cells, arrestin 3 did seem to play an important role, suggesting that cellular migration occurs via arrestin-dependent signalling. Data analysis confirms that migration of cells can occur independently of calcium release as discussed in chapter 4 [142].

Arrestins have recently been described by literature as essential regulators of cell migration either towards or in the presence of a chemical ligand by interacting with a cell-surface receptor. Their role in migration of cells is poorly understood; whether their role in migration of cells reflects receptor desensitisation or localised scaffolding of cytoskeletal signalling molecules (figure 5.18) [18,81].

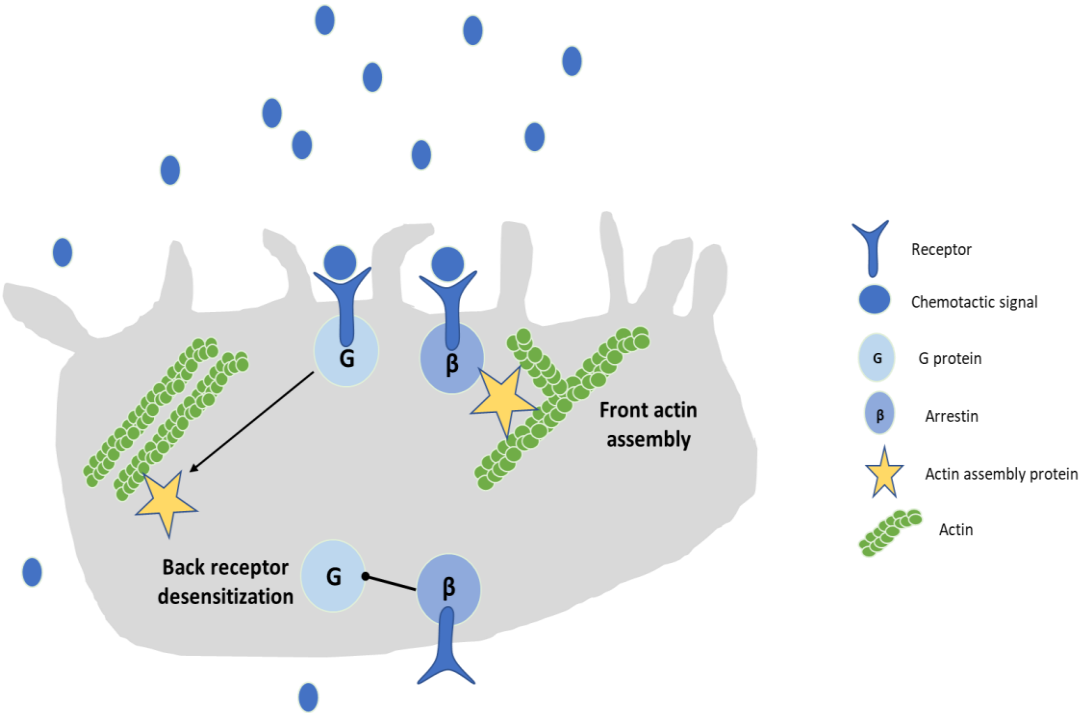


Figure 5.18 (adapted from DeFea *et al.* (2007) [81]): Localised actin assembly versus receptor desensitisation. Receptors at the front of the cell are exposed to a higher concentration of chemokines. Arrestins may preferentially uncouple receptors from their cognate G-protein in the back of the cell while G-protein signalling simultaneously persists in the front. Another theory is that arrestins may also scaffold cytoskeletal proteins at the front of the cell, leading to localised actin assembly events.

Actin cytoskeletal reorganisation and cell migration require arrestins to regulate downstream signalling, which eventually lead to these two processes [81]. By regulating receptor internalisation and by scaffolding of signalling molecules, arrestins facilitate gradient sensing and cytoskeletal reorganisation which leads to cell migration [18], suggesting that arrestins use these two functions to orchestrate cellular response to chemotactic signals. Actin polymerisation from monomers is a spontaneous but slow process and has been shown to be the driving force behind directed cell movement and can be regulated by various proteins [18,81]. Based on visual assessment, arrestins did not seem to have an obvious effect on actin polymerisation in MCF-7 cells. Further experiments could be done to confirm the involvement of arrestins in actin polymerisation upon CXCL12 activation via CXCR4 receptors such as quantifying fluorescence in flow cytometry or using different and larger sized cells to visualise changes in actin upon CXCL12 activation.

The internalisation of chemokine receptors occurs after the ligand binds to the receptor, so depending on the percentage of receptors being activated, internalisation may reduce the level of receptor expression at the membrane and therefore change functionality [313]. Based on arrestins 'classical' role, previous literature has indicated that they are mediators of receptor desensitisation and internalisation [80,81,380], but this doesn't mean that role of arrestins in chemokine receptor signalling can be generalised as each receptor may utilise arrestins differently in the process of chemotaxis [18]. Based on our preliminary data, overexpression of arrestins did not have an effect on internalisation of CXCR4 receptors upon 15 nM CXCL12 stimulation. There are two explanations for this: receptor turnover and differential signalling in response to ligand multimers. CXCL12 monomers and dimers elicit different signalling where CXCL12 monomers have the ability to promote arrestin recruitment and evoke arrestin-dependent signals, whilst the dimers do not [18]. Therefore, in addition to simply promoting receptor turnover, arrestins may mediate signalling at lower CXCL12 concentrations while at higher concentrations, their signal may be dampened. This could explain why there was not an increase in internalisation of CXCR4 receptors upon CXCL12 stimulation in Jurkat cells overexpressing arrestins. However, it is difficult to draw a conclusion from this data at this stage as more experiments are needed to confirm the reliability and reproducibility of these results.

MAPK networks are critical for the transmission of extracellular signals into appropriate intracellular responses. Once activated, MAPKs can phosphorylate a wide array of intracellular targets that include cytoskeletal elements, membrane transporters, nuclear pore proteins, transcription factors and other protein kinases [80,382] . Arrestins have been shown to serve as scaffolds for numerous signalling networks; arrestin-dependent signalling was distinct from its role in internalisation and is thought to be responsible for ligand-biased signalling observed through GPCRs [81].

Overexpression of arrestin 3 resulted in an increase in cellular migration, which was further confirmed by siRNA knockdown of arrestin 3 expression, proving that arrestin 3 is the important arrestin subtype that may be involved in migration of cells. Knockdown of arrestins via siRNA is a useful investigative tool and clearly shows the importance of different arrestin subtypes in CXCL12-induced migration via CXCR4.

Simultaneous knockdown of arrestin 2 and arrestin 3 further confirmed that arrestin 3 is the main subtype in CXCL12-induced migration as there was not further reduction in the number of migrating cells when both arrestins' subtypes were knocked down. This data supports previous findings, where arrestin 3 is critically involved in CXCR4-mediated chemotaxis [145,182], and according to literature, this is mediated by its enhancement of p38 MAPK activation [182]. However, we could not show that there definitely was a knockdown of arrestins due to the lack of availability of reliable arrestin antibodies. Other methods should be considered for future work, which includes RT-PCR to check for the presence of the mRNA.

The potent MEK1/2 inhibitor, PD98059, was used to investigate if ERK1/2 phosphorylation is needed for migration or a consequence of scaffold formation that is unrelated to migratory responses. The data suggested that CXCL12-induced migration via CXCR4 activation is sensitive to ERK1/2 inhibition and that migration of cells via ERK1/2 MAPK pathway is not arrestin-dependent. This data does not support the theory of arrestin as a scaffold for ERK1/2 MAPK pathway since there weren't any differences between mock transfected cells and Jurkat cells that overexpressed arrestin 3, but this might only mean that arrestin 3 and GPCR scaffold complex is not applicable in downstream ERK1/2 MAPK signalling that results in CXCL12-induced migration.

However, there were different effects observed between mock and pArr3.EGFP transfected Jurkat cells when SB203580, a potent p38 MAPK inhibitor was used. We observed that overexpression of arrestin 3 in Jurkat cells had less inhibition of migration of cells, which meant there were more cells migrating in pArr3.EGFP transfected cells compared to the control mock transfected Jurkat cells. This data suggests that arrestin-mediated signalling occurred, and this can be interpreted as evidence to support arrestin scaffold signalling. It is implied that when there was an overexpression of arrestin 3 in Jurkat cells, there are more arrestin 3-CXCR4 receptors complexes formed to scaffold p38 MAPK activation cascades [382]. The p38 MAPK has been associated with α-chemokine receptor mediated arrestin-dependent signalling [383]. Therefore, it is important to determine the effect of p38 inhibition in CXCR4 mediated chemotaxis on Jurkat cells. The effects of p38 inhibition clearly show that in CXCL12-induced migration via CXCR4, these proteins are activated by arrestins and that their kinase function is required in CXCR4 mediated chemotaxis.

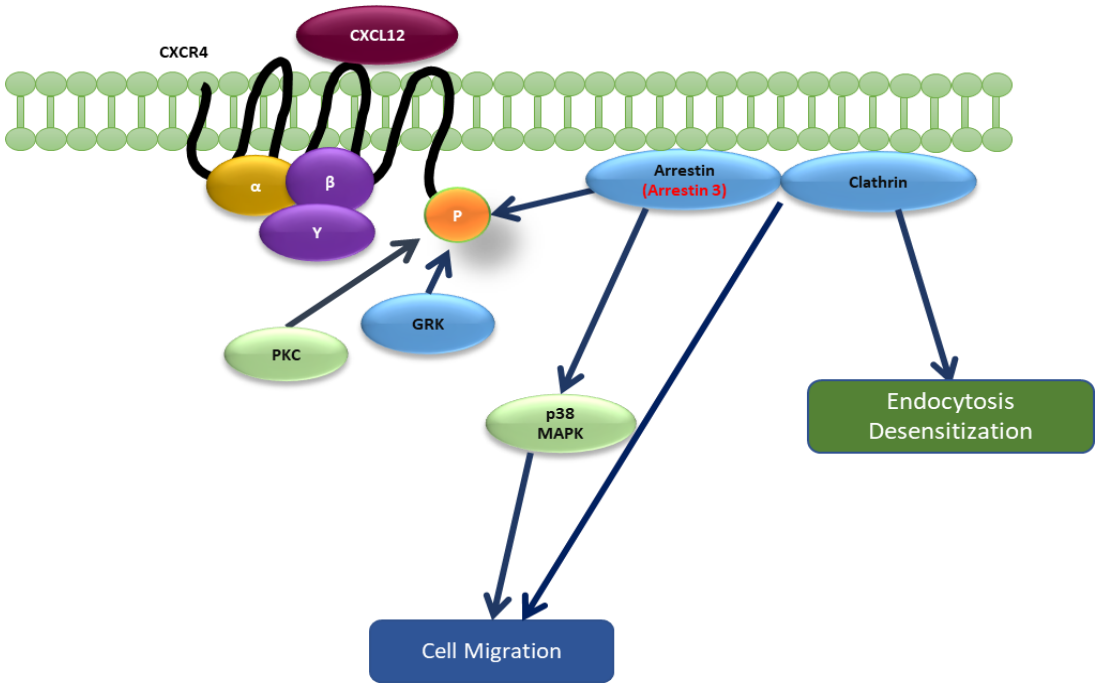


Figure 5.19: An overview of chapter 5 results. Cellular signal transduction involves highly coordinated cascades of events. Data indicated that arrestins may be involved in p38 MAPK pathway, and that they may act as signal transduction scaffolds or is dependent on arrestin signalling.

These results outline a picture of CXCL12-induced migration via CXCR4 and from them, it was demonstrated that arrestin 3 is the subtype that is most likely involved in migration of cells (figure 5.19). It is important to identify which signalling proteins further downstream are shared with canonical migratory signalling, as there is a unique signalling axis, dependent on both chemokine receptor and cell type. In order to develop drugs targeting chemokine receptors, it is important to consider biased signalling and understand which residues are important for ligand binding and subsequent receptor interaction with different intracellular effector molecules. Biased signalling, which results in activation of one of several different pathways, should be considered and it can be divided into three distinct forms – ligand bias, receptor bias, and tissue or cell bias [260]. Most biased signalling is differentiated into G-protein dependent and arrestin-dependent signalling.

The presented data supports a role for arrestin-dependent signalling; however, it is still unclear the exact nature of arrestin in signal transduction, and there is a need to

study these relationships clearly at recruitment and signalling level. These observations leave unanswered questions: the relationship and associated machinery between activated receptor and arrestin as well as the mechanism of arrestin-dependent assembly with different downstream effector molecules. Hence, further studying and understanding how GPCR signalling molecules engage in signal transduction and activate signalling pathways is important.

Another factor that should have been considered in these experiments is the presence atypical chemokine receptor, ACKR3 (also known as CXCR7) receptor in Jurkat cells. ACKR3 is also a CXCL12-binding receptor. ACKR3 functions as a scavenger receptor for CXCL12 and it has been shown that ACKR3 suppresses processes, including tumour growth, survival and proliferation of breast cancer cells, in multiple organs and tissues [384,385]. Melo *et al.* (2014) has demonstrated that ACKR3 is highly expressed in Jurkat cells and that it can potentiate CXCR4 response to CXCL12 [386]. Their studies suggested that the presence of ACKR3 may be important in potentiating the migration induced by CXCR4/CXCL12 signalling and this may be due to the heterodimerization of both receptors [386]. In the case of the CXCR4-ACKR3 heterodimer, it has been hypothesised that there is an increase in arrestin-dependent signalling, which resulted in enhanced cell migration [387]. In co-expression of CXCR4 and ACKR3, the CXCR4-ACKR3 heterodimer complex recruits arrestins, which resulted in biased signalling towards arrestin-dependent signalling over G-protein signalling [387,388]. The presented data in this chapter did not take into account the presence of ACKR3, hence further work is needed to be done to establish the relationship between CXCR4 and ACKR3 in CXCL12-induced signalling before drawing up definite conclusions.

Bioluminescence resonance energy transfer (BRET) can represent a useful investigative tool for future work; to understand the role of arrestins more in-depth in terms of structure and relationship of arrestins with other different effector components in downstream signalling pathway. Arrestins can interact with downstream effectors in different modes, so structural studies are important in understanding how arrestins transmit signals encoded in the receptor to these effector molecules [193].

Identifying and mapping the pathways that are involved would be crucial to understanding downstream signalling that leads to migration of cells, and which components are important for signal transduction.

5.5. Chapter conclusions

The final conclusions to be drawn from this chapter are:

1. The role of internalisation and calcium release may be arrestin-independent, but this is not the case in studying the role of arrestins in CXCL12-induced migration of cells. Changes in level of arrestin expressions seems to affect migration of cells.
2. Arrestin 3 is required in CXCl2-induced migration of cells, showing that arrestin 3 seems to be important for migration in both suspension Jurkat cells and adherent MCF-7 cells.
3. p38 MAPK pathway may be arrestin 3-dependent signalling upon CXCL12 activation of CXCR4 receptor.

CHAPTER 6: Final discussion and thesis conclusions

6.1. Final discussion

In this thesis, we characterised CXCL12-induced migration via CXCR4 in different cell lines with the aim of understanding roles of downstream effectors, particularly of PKC and arrestins, in the migration of cells and whether they make useful targets to treat CXCR4-mediated chronic immune and metastatic disease. Overall, the conclusions from this research are as follows:

1. MCF-7 and Jurkat cells are good model systems to study the CXCR4/CXCL12 signalling axis, and CXCL12 is the most appropriate chemokine to study the biology of CXCR4 in downstream signalling cascades leading to migration of cells.
2. The N-terminus of CXCL12 is important in initiating signalling events leading to migration of cells. The CXCL12 N-His Tag induces intracellular calcium signalling but not migration of cells. This suggests that CXCL12 N-His Tag is also a partial agonist of CXCR4.
3. PKC activation is essential for the migration of adherent breast cancer cells, MCF-7 cells, as PKC inhibition affects CXCL12-induced migration in MCF-7 cells antagonistically. This is not true of suspension leukemic T-lymphocytes, Jurkat cells, which suggests that there is a difference in the usage of PKC in different cell types and that cellular background is important for distinct signalling pathways upon CXCR4 activation by CXCL12. Therefore, a generalisation of how CXCR4 induces migration in different cell types and species should be avoided as there may be presence of bias at tissue level.
4. Although CXCR4 internalisation and calcium release may be arrestin-independent, CXCL12-induced migration of cells via CXCR4 depends on arrestins, as changes in level of arrestin expressions affects migration of cells.
5. Arrestin 3 is required for CXCL12-induced migration of cells, but not the arrestin 2 subtype, in both MCF-7 and Jurkat cells. The requirement of arrestins for cellular migration in downstream of CXCR4 suggests that arrestin 3 plays a key role in mediating CXCL12-induced cell migration.

6. p38 MAPK pathway may be arrestin 3-dependent in CXCR4/CXCL12 signalling axis but a specific role for arrestin-dependent sequestration of MAPKs in chemotaxis has not been shown.

As stated in the introduction, the aim of this thesis is to investigate the activation of signalling molecules in the downstream signalling cascades in CXC- chemokine receptors. This was to identify potential targets to CXCL12-induced migration to by-pass the problems with receptor redundancy and transactivation. There is a need for therapies to block cell migration since there is a major involvement of chemokines and chemokine receptors in both chronic inflammatory and cancer metastasis [86,389]. GPCRs have been proven to be attractive targets for therapeutically potential in drug discovery over the decades, and more than 30% of all prescription drugs are directed at fewer more than 50 known receptors [390]. Chemokine receptors are part of the GPCR family, and for the last two decades, many pharmaceutical companies have been trying to develop drugs blocking specific chemokine receptors. Chemokine receptors were considered to be highly promising drug targets for inflammatory and immunological diseases but there are only few instances that have reached the approval for clinical use; Maraviroc (CCR5 antagonist), Plerixafor (CXCR4 antagonist) and Mogamulizmab (anti-CCR4) are amongst the current potential clinical applications of individual chemokine receptors [315]. There are several possible reasons why this is the case; the intrinsic functional redundancy in the chemokine system, possible cross-talk between chemokine receptors and signalling are dependent on cells and species specificity may be some of the reasons to why chemokine system as potential drug targets still remains unexplored [19,315,391,392].

The interaction between CXCR4 and CXCL12 has been proposed to occur through a two-step process [270,327], and the initial interaction between RFFESH motif (residues 12-17) of CXCL12 and residues 2-36 of CXCR4 are believed to result in a conformational change in the receptor [393].

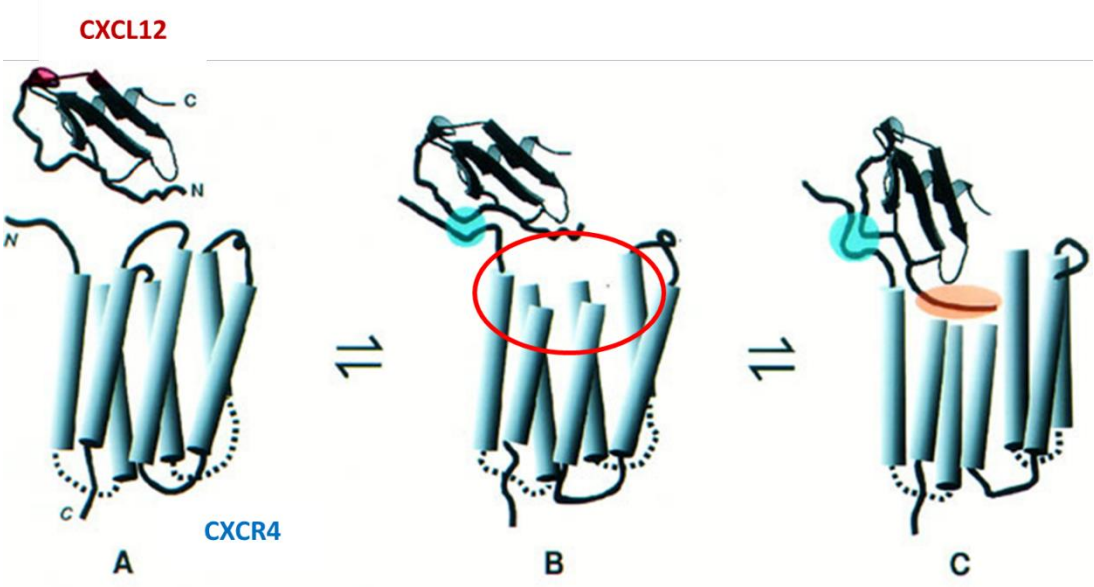


Figure 6.1 (adapted from Crump et al. (1997) [327]): A schematic model for interaction of CXCL12 with CXCR4. (A) Receptor CXCR4 and ligand CXCL12 prior to any interaction between the two. (B) Interaction of CXCL12 RFFESH loop (site 1) with the N-terminal segment of CXCR4. The contact region is shown in blue. Conformational change of the receptor occurred, exposing the binding groove of the receptor (circled in red). (C) N-terminal region (site 2) of CXCL12 bound in groove at the top of the helices (shown in orange).

RFFESH motif in the loop region function as a docking site for CXCL12 receptor binding [327] and it is important for the initial interaction with CXCR4 in the two-step model of chemokine-receptor activation as mutation in this motif significantly decreased chemotactic activity [270,394]. The RFFESH formed a receptor binding site and is very well defined in CXCL12 [327]. This explains why CXCL12 N-His Tag still induces signalling events as initial binding can still occur due to the presence of RFFESH motif in CXCL12 N-His Tag and resulting in conformational changes of the receptor, thus activating the receptor (figure 6.1B).

In figure 6.1C, the binding of the N-terminal region results in activation of the receptor. This conformational change facilitates interaction between the first eight amino acids of CXCL12 and an exposed binding pocket in CXCR4 [257,329] (figure 6.1C). Crump *et al.* (1997) demonstrated the importance of first N-terminal 8 residues as they formed an important receptor binding site and modification to Lys-1 and Pro-2 resulted in loss of activity but generated CXCL12 antagonists [327]. This observation was confirmed by conclusion 2, as interaction of CXCL12 N-His Tag (15 amino acids fused at the N-terminus) with CXCR4 resulted in antagonistic effects at increasing concentrations.

This data also indicates CXCL12 N-His Tag may have effects that are analogous to CXCL12 dimers. CXCL12 may oligomerize at increasing concentrations and dimeric forms have been found to act as a partial agonist, which stimulates calcium mobilisation but has no chemotactic effect, and they can also act as a selective antagonist that blocks chemotaxis induced by the monomeric form of CXCL12 [66,324]. It is hypothesised that CXCL12 N-His Tag may be a partial agonist, like dimeric CXCL12.

Activation of the receptor by chemokine ligand leads to signalling via heterotrimeric G-proteins, which ends with rapid phosphorylation of the receptor via GRK2 [142]. Recent studies [125,141,395] have shown that PKC may play a role in phosphorylating receptors and therefore lead to the desensitisation of receptors, triggering binding of arrestins and consequently, internalisation of the receptor [66,396]. Oppermann *et al.* (1996) [343] have shown that both GRK and PKC are equally important in phosphorylation of receptors. Hence, PKC has the potential to phosphorylate ligand bound GPCR and multiple other receptors in a heterologous manner [141].

Turning now to the experimental observations on the role of PKC, PKC activation is not essential for the migration of suspension leukemic T-lymphocytes, Jurkat cells, but the blockade of PKC activation using small molecules antagonists inhibits migration of adherent breast cancer, MCF-7 cells. Instead of inhibiting the migration of Jurkat cells, antagonising PKC activation in Jurkat cells seem to have an opposite effect than what was observed in MCF-7 cells, resulting to an increase in cell migration instead of an inhibition. It is possible, therefore, that PKC has an important role in desensitisation of the receptor in Jurkat cells. Hence, when PKC is inhibited, phosphorylation of receptor is blocked and causing prolonged activation, which may be the explanation to why an increase of migration occurs.

This data, which demonstrates there is a difference in the usage of PKC in different cell lines, also supports the hypothesis that signalling is dependent on cells and species (conclusion 3). This hypothesis is supported by other literature [19,20,313,315], hence, generalisation of how chemokine receptors in different cell types and species should be avoided. MCF-7 cells need the catalytic activity of PKC, thus, small molecule antagonists as well as knockdown of PKC prevented migration. In Jurkat cells, it seems

plausible that it is the functionality of the other PKC domains which is implicated in cell migration and not the kinase activity. However, the research did not consider that there is a possibility that different PKC isoforms are required in migration of different cell lines. The antagonists used inhibit conventional (PKC-α, β1, β2, γ) and novel (PKC-δ, ε, η, θ) types of PKC isoforms but not atypical PKC isoforms (PKC-ι, ζ). There are variety of studies [341,348,397] that demonstrates roles of different PKC isoforms are important for cancer cell migration.

After preliminary experiments to establish the role of PKC cell migration, studying the role of arrestins is the next step to investigate in the CXCR4/CXCL12 signalling axis as arrestins have been shown to be involved in cell migration. Arrestins bind to the phosphorylated receptor, and play a role in desensitisation of the receptor, thus terminating the cell signalling. Traditionally, arrestins are known to be mediators of receptor desensitisation , but recently, arrestins have emerged as key regulators of directed cell migration [81]; by directly binding and regulating proteins involved in actin reorganisation or by scaffolding signalling molecules involved in cytoskeletal reorganisation [18].

CXCL12-induced responses are sensitive to Src [398–400], p38 MAPK, ERK1/2 MAPK inhibition [229,286,323,353] and are arrestin 3-dependent (conclusion 6). This provides strong evidence that arrestin 3 may be involved in signalling related to the migration of cells, supporting the observations from Fong *et al.* (2002) [191] where knockdown of arrestin 3 abrogates migration downstream of CXCR4/CXCL12 signalling axis. Arrestin 3 may also be involved in the p38 MAPK pathway (conclusion 7) but not in the MEK/ERK MAPK pathway as increasing expression levels of arrestin 3 affect p38 MAPK inhibition (by small molecule antagonist, SB203580), resulting in significantly less inhibition of cells via the p38 MAPK pathway. Arrestin 3 does not seem to be involved in the MEK/ERK MAPK pathway as increased expression levels of arrestin 3 in Jurkat cells did not affect the inhibition by PD98059 (MEK inhibitor).

A possible explanation to this observation is that arrestins serve as molecular scaffolds for the p38 MAPK pathway (figure 6.2a) and this hypothesis is supported by several publications in the current literature, where it has been shown that arrestins act as scaffold for numerous signalling proteins including MAPK [81,179]. However, further

experiments need to be done to investigate the involvement of other signalling molecules in the p38 pathway and to see if there is direct interaction between affected signalling molecules and arrestins. With that hypothesis, it is possible that arrestin 3 acts as a scaffold indirectly to MEK activation (figure 6.2b). Hence, increasing expression level of arrestin 3 does not affect MEK inhibition (by PD98059) and that there is a significant decrease in migration of Jurkat cells in the CXCR4/CXCL12 signalling axis.

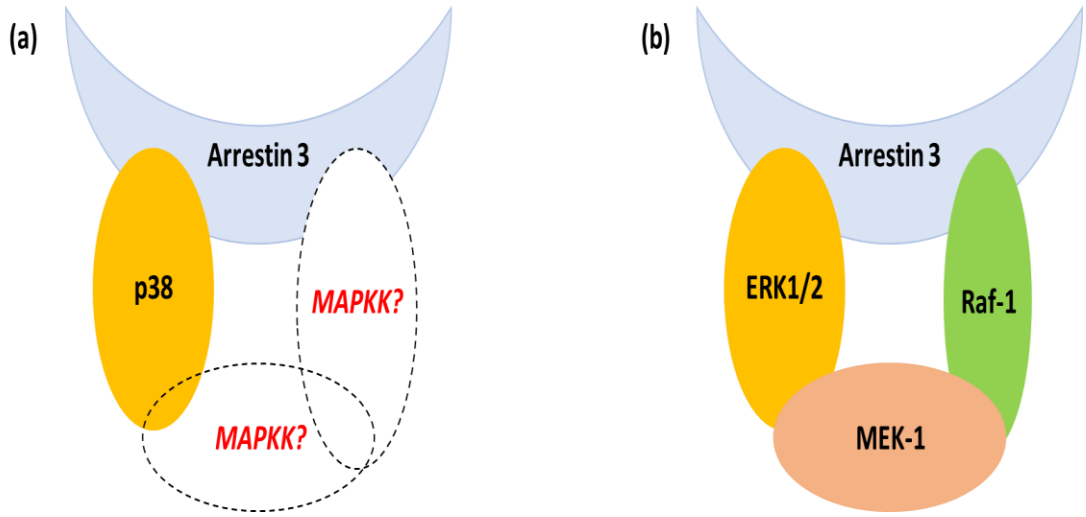


Figure 6.2 (adapted from DeWire et al. (2007) [179]): Arrestin scaffolds for signalling. (a) A schematic diagram to illustrate arrestin scaffold for p38 activation. There is a possibility that p38 bind arrestin 3 directly and thus, changes in expression level of arrestin 3 affects p38 inhibition (by SB2032580), resulting in reduction of inhibition of cellular migration. Further experiments need to be done to investigate other MAPKK signalling molecules that bind to arrestins directly or indirectly (depicted by dotted lines). (b) Arrestin scaffold for ERK activation. ERK 1/2 and Raf-1 bind arrestin 3 directly, and MEK-1 binds indirectly. It is not known which residues of arrestin 3 bind ERK and Raf-1.

Numerous receptors that require arrestins for migration also promote arrestins' dependent association with MAPKs [180,182,375], but the consequences of these signalling events are still poorly understood. These experimental observations highlight the potential for inhibitors of well-validated signalling targets, such as PI3K and PKC, to be ineffective against certain diseases where CXCR4/CXCL12 signalling occurs [286].

Arrestin, GRK [146,191] and PKC may be involved in migration but their suitability as targets to block migration for therapeutic potential is low due to their ubiquitous involvement in GPCR signalling and the high possibility of potential pathological signalling by preventing desensitisation of receptors. Identifying players in arrestin-dependent signalling to migration is likely to offer higher validity targets. It is clear that arrestin 3 is involved in CXCR4/CXCL12 signalling axis (conclusion 6) and there is a possibility that it may be via MAPK pathway (conclusion 7). However, a specific role for arrestin-dependent sequestration of MAPKs in chemotaxis has not been shown.

Table 6.1 lists the protein targets described in this thesis regarding their validity as therapeutic agents against CXCL12-induced migration via CXCR4. Recently, an atypical second CXCL12 receptor, ACKR3 has been described to be essential in signalling to migration by acting as a CXCL12 scavenger to regulate local chemokine availability and directly modulating arrestin mediated signalling cascades [401]. This complicates the CXCR4/CXCL12 signalling network. Therefore, there is a need to investigate and dissect the contributions of CXCR4 and ACKR3 to the different steps of metastasis as both CXCR4 and ACKR3 have been found to enhance tumour growth and metastasis formation by binding to CXCL12 [402].

Targeting chemokine receptors directly may represent a poor target choice for anti-migratory therapy but it is advantageous to identify key players amongst signalling molecules and understand downstream signalling transduction upon chemokine receptor activation to allow successful treatment of patients. Targeting arrestin directly does not represent a valid strategy for two reasons. Firstly, all GPCRs require arrestin for regulation and inhibiting arrestin may result in a decrease in arrestin-dependent signalling, where it occurred, but would also lead to a reduction in desensitisation in GPCRs in 'classical' G-protein mediate signalling. This could lead to severe negative effects physiologically. Secondly, there are no small molecule inhibitors of arrestin at present, which adds a further element of complexity with regard to the *de novo* discovery of small molecule arrestin antagonists.

Table 6.1: Validity of proteins as targets in CXCL12-induced chemotaxis.

Protein	Target validity	Rationale	Refs
CXCR4	Moderate	AMD3100 acts as CXCR4 antagonist (or partial agonist) and an allosteric agonist of ACKR3. Blockade of chemokine receptor signalling is complex due to signalling redundancy and possibility crosstalk between receptors. Another complexity is that ACKR3 and CXCR4 can oligomerize either with one another of its own family or each other.	[270,403,404]
PKC	Inconclusive	PKC inhibition using pan-isoform PKC inhibitors have different effects in different cell lines. Further experiments need to be done as PKC has an unclear role in CXCL12-induced migration. PKC isoform specificity should also be taken into account.	Chapter 4
Arrestin	Moderate, but needs to be under serious consideration	Other than arrestin-dependent signalling and arrestin acting as molecular scaffolds, arrestin is required for desensitisation of all GPCRs and therefore, it is most likely to impact other GPCR signalling adversely. Arrestin subtype specificity would also need to be considered.	Chapter 5
p38 MAPK	Good	p38 MAPK inhibition is effective in blocking CXCL12-induced migration of Jurkat cells but may lack specificity between receptor types. It is arrestin 3- dependent but involvement of arrestin needs to be investigated further.	Chapter 5
MEK/ERK MAPK	Good	MEK inhibition is effective in blocking CXCL12-induced migration of Jurkat cells and is not arrestin 3-dependent signalling.	Chapter 5

6.2. Future work

As discussed in the introduction (chapter 1), the aim of this thesis is to understand the role and interactions of proteins of interest in CXCL12-induced signalling events upon CXCR4 activation. This was achieved to a certain extent, but involvement of ACKR3 was not considered in this thesis. Signalling stimulated by CXCL12 via CXCR4 was shown to occur through arrestin-dependent signalling and it seems to also involve p38 MAPK in migration of cells. There are limitations to the conclusions as there may be involvement of ACKR3 on CXCL12/CXCR4 signalling axis and how conformational changes in arrestins upon receptor activation contribute to signalling is still poorly understood. The conclusions drawn from this thesis allows more in-depth proteomic characterisations to be undertaken. Involvement of arrestin 3 in CXCL12-induced migration has been established, therefore, the future work based on this thesis falls into these areas:

1. Interactions of ACKR3 in CXCR4/CXCL12 signalling axis.
2. Involvement of ACKR3 in arrestin-dependent signalling in CXCR4/CXCL12 axis.
3. Further investigations in different PKC isoforms affecting cell migration.
4. Investigations in conformational changes in arrestins upon CXCR4 activation contribute to its signalling and endocytic functions.

The first two areas will be complex as it involves experiments using ACKR3 antagonists, which can only obtained from Chemocentryx and it is not for sale [405]. Another approach that could be considered is CRISPR/Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats) [406]. By knocking out ACKR3 gene using CRISPR/Cas9 system, it is possible to look at CXCR4 in isolation and to study the importance of each receptor in cells.

ACKR3 expression seems to be enhanced during pathological inflammation and tumour development [404]. Emerging evidences suggest that ACKR3 can be an attractive therapeutic target for autoimmune diseases and cancer [402,407]. There is suggestion that ACKR3/CXCR4 heterodimerization, arrestin-mediated signalling and modulation of CXCL12 responsiveness by ACKR3 has an impact on understanding the

roles of CXCL12 biology in health and disease [404]. Hence, there is an importance in understanding the role of ACKR3 in CXCL12/CXCR4 signalling axis. Obtaining ACKR3 antagonists would allow investigations of relationship between arrestins, CXCR4 and ACKR3 and how each component contribute to CXCL12-induced signalling. Area 3 will involve further small molecule validation using other inhibitors, and also involve additional siRNA experiments to understand the role of different PKC isoforms in CXCL12-induced signalling. Investigations using small molecule inhibitors and other cell lines can further establish the role of PKC.

Finally, area 4 will involve studying protein-protein interaction in real time using bioluminescence resonance energy transfer (BRET). BRET requires proteins of interest and suitable controls that are labelled with either a donor or acceptor molecule. BRET involves nonradiative (dipole-dipole) transfer of energy from a donor enzyme to a suitable acceptor molecule after substrate oxidation [408]. The transfer of excited-state energy is inversely proportional to the sixth power of the distance between donor and acceptor dipoles, and this distance is comparable to the dimensions of biological macromolecular protein complexes [409]. Thus, this makes BRET an appropriate method for assessing protein-protein interactions. There are three BRET derivations; $BRET^1$, $BRET^2$, and eBRET. The differences between them lies in use of different substrates and the donor and acceptor emission peak separation, where the more they separate, the easier that the donor and acceptor emissions to be distinguished, thereby reducing the background [408].

The BRET technique is most suited to investigate ligand-modulated interactions as changes in BRET signal after reagent addition provide good evidence for interaction specificity, and changes in BRET signal may represent an increase or decrease in the number and/or rate of interactions between the proteins of interest [410]. The proteins of interest are genetically fused to the bioluminescent donor or fluorescent acceptor, and a suitable substrate is selected for the appropriate combination for BRET assay. The BRET technology uses the combination of a 35 kDa *Renilla Luciferase* (RLuc) as energy donor, a 27 kDa variant of GFP as energy acceptor, and appropriate substrate for different BRET derivatives. In this case, CXCR4 receptor is fused to RLuc3 as donor molecule and human arrestin 3 is fused to GFP10 as acceptor molecule; these plasmid constructs were gifted from Professor N. Heveker (University of Montreal). With these

constructs, DeepBlueC (coelenterazine 400a) is used as a substrate to perform the BRET^2 assay. BRET detection after addition of substrate is done with the use of two distinct filters; one filter transmits the 'short-wavelength emission' that should consist of the donor emission of short wavelengths (excluding as much of the acceptor emission) and the other filter transmits the 'long-wavelength emission' that consists of the acceptor emission (if present) in addition to the donor emission of longer wavelengths [408]. In BRET^2 assay, the example filters of 'short-wavelength emission' is 370-450 nm and 'long-wavelength emission' is 500-530 nm [410]. BRET signal is analysed with this formula below:

$$\text{BRET signal} = \frac{'\text{long} - \text{wavelength emission}'}{'\text{short} - \text{wavelength emission}'} - \frac{'\text{long} - \text{wavelength emission for donor only trasnfected cells}'}{'\text{short} - \text{wavelength emission for donor only transfected cells}'}$$

We started setting up the necessary experimental set-up in our laboratory and had some encouraging preliminary results, but more optimisation is needed to be done as we could not obtain clear confirmation that there was a BRET signal between CXCR4-RLuc3 and h-arrestin 3-GFP10 upon CXCL12 stimulation. A difference in emission readings between untransfected, transfected and donor-only controls were detected but the BRET signal was not as high as what was obtained in comparable experiments published in other labs [410]. Below is a table showing the possible problems and solutions to help in optimisation for future work (table 6.2).

Table 6.2: Troubleshooting table for BRET^2 assays [323,409,410]

Problem	Possible reason	Solution
Cells were lost and easily detached after transfection, despite extreme care.	Cells that were usually used in laboratory are MCF-7 cells and Jurkat cells. These cells were transfected prior to BRET assays: • MCF-7 cells - Chemically transfected and may not be sufficiently adherent. • Jurkat cells - Electroporation transfected and cells are usually more fragile and less viable after electroporation transfected.	• Consider using different plastics or coating wells (e.g. with poly ($_L$-lysine)). • Consider using different cell lines that are easily transfected, such as HEK-293 cells.
There were low relative luminescence or fluorescence counts	• There is poor protein expression. • The cell number is low. • Substrate is not viable. • Reducing agent present. • Instrumentation (CLARIOstar, BMG LABTECH) is not functioning correctly, and filters available are not suitable.	• Optimise and consider an alternative transfection strategy. • Increase cell concentration and consider changing cell lines. • Consider using new aliquot of substrate and ensure dilution of substrate stock to a working concentration in assay buffer is accurate. • Ensure buffers do not include reducing agents such as ascorbic acid and also, ensure that it was stored appropriate and made up accordingly to perform BRET^2 assays. • Familiarise the use of instrument with protocols available and the help of Clariostar technicians. • Check instrument setup and consider recalibration. Ensure that suitable filters were available for BRET^2 assays.

Studying the relationship between chemokine receptor and arrestins upon activation would help in understanding the conformational changes in arrestins upon CXCR4 activation, and the BRET technology would enable us to study protein-protein interactions in live cells where they are expressed in a near-physiological environment in the correct cellular compartment [410]. Once that has been established, studying protein-protein interactions in the presence or absence of small molecule antagonists is possible, and hence, further characterising the role of different signalling proteins in arrestin-chemokine receptor interaction. As a whole, this future work, in addition to the research presented in this thesis, has the potential to enhance productivity in area of pharmacology, particularly CXCR4/CXCL12 signalling axis, and has the possibility to achieve therapeutic potential.

CHAPTER 7: References

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