

**Pharmacological insights into C-C motif
chemokine receptor 5 mediated
chemotaxis**

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ABSTRACT:

Aim: Despite being well validated as a therapeutic target, no chemokine receptor antagonists to be used as therapeutic agents in inflammatory or metastatic disease have made it to market. This is in part due to receptor redundancy, but also a lack of understanding with regard to cytoplasmic signal transduction linking activated chemokine receptors to chemotaxis. Resolving signal transduction pathways in model chemokine receptor systems may allow intracellular drug targets to be identified, bypassing the difficulties associated with extracellular chemokine receptor blockade.

Methodology: Experimentation was undertaken in THP-1 monocytes expressing the chemokine receptors CCR5 and CCR1 and in stably CCR5-transfected HeLa and CHO cell lines. Small molecule inhibition and protein overexpression was used before chemotaxis and calcium release assays to measure cellular responses. Immunocytology was used to determine the effect of protein blockade on receptor internalisation, protein localisation and the formation of cellular structures associated with migration. Experiments were also performed in activated primary tissue for comparative analysis and validation of results in normal human tissue.

Results: A systematic blockade of signalling proteins by small molecule means revealed that G $\beta\gamma$, ERK1/2, p38 and PI3K are not required for CCL3 stimulated monocyte migration. GRK2 and PKC inhibition along with internalisation blockade showed antagonistic effects on the ability of cells to migrate, suggesting arrestin dependent signalling was involved in chemotaxis. Inhibition of dynamin, Grb2 and non-receptor tyrosine kinases were equally effective at blocking migration in THP-1 cells but less effective at blocking CXCL11 stimulated migration in activated PBLs.

Conclusions: This study has shown that CCL3 stimulated chemotaxis through CCR5 does not occur through typical G-protein mediated signalling, but maybe therapeutically accessible by inhibition of dynamin and Grb2. Additionally the differences in dynamin inhibitor efficacy suggest that the production of migration specific dynamin inhibitors may be possible. Overall the research in this thesis has identified novel targets for therapeutic intervention in diseases where dysregulation of chemokine receptor mediated migration are causative.

ABBREVIATIONS:

Ab	Antibody
ANOVA	Analysis of variance
β ARK	β -Adrenergic receptor kinase (GRK2)
BSA	Bovine serum albumin
CCL	C-C motif chemokine receptor ligand
CCR	C-C motif (β) chemokine receptor
CHO.CCR5	Chinese hamster ovary cells, CCR5 stably transfected
c-Src	Cellular sarcoma non-receptor tyrosine kinase
CXCL	C-X-C motif chemokine receptor ligand
CXCR	C-X-C motif chemokine receptor
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
EC ₅₀	Concentration at which 50% of an effect occurs
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal-regulated kinase 1/2
FCS	Foetal calf serum
G α	G-Protein α subunit
G $\beta\gamma$	G-Protein $\beta\gamma$ subunit
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-Protein coupled receptor
Grb2	Growth factor receptor-bound protein 2
GRK	G-Protein coupled receptor kinase
GTP	Guanosine triphosphate
HeLa.RC49	Henrietta Lacks cell line, CCR5 stably transfected
HIV	Human immunodeficiency virus
IC ₅₀	Concentration at which 50% inhibition occurs
IL-2/6	Interleukin 2/6
JAK	Janus kinase
Lyn	Lck/Yes-related novel protein tyrosine kinase
MAPK	Mitogen activated protein kinase
p38	p38 mitogen activated protein kinase
PBL	Peripheral blood lymphocyte

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PH	Pleckstrin homology
PI	Phosphoinositide
PI3K	Phosphoinositide-3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PRD	Proline rich domain
Pyk2	Protein tyrosine kinase 2
Ras	Rat sarcoma GTPase
Rho	Ras homology GTPase
RME	Receptor mediated endocytosis
ROCK	Rho kinase
RPMI	Roswell Park memorial institute medium
RTK	Receptor tyrosine kinase
SEM	Standard error of the mean
SFK	Src family kinase
SH2/3	Src homology domain 2/3
siRNA	Small interfering ribonucleic acid
SOS	Son of sevenless
STAT	Signal transducer and activator of transcription
TG	Thapsigargin
THP-1	Acute monocytic cell line THP-1
WT	Wild type

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CHAPTER 1: Introduction

1.1 Introduction

The chemokine receptor CCR5 was discovered as a co-receptor for the human immunodeficiency virus 1 (HIV1) entry in to human macrophages [1-3]. Accordingly, large amounts of research were undertaken to characterise CCR5-HIV interaction [4-9] with the hope, that sufficient understanding would allow the production of effective anti-HIV prophylactics to combat the ever-increasing global prevalence of infected individuals. HIV entry inhibitors were eventually developed [10] with one, maraviroc, making it to market [11]. Almost as an aside to the research directly involving HIV, a great deal of information had been gained about CCR5 structure and function [4, 12-15]. This chemokine receptor, after all, must have some physiological function outside of HIV viral entry. The discovery that large numbers of individuals carried the CCR5 Δ 32 mutation that rendered the receptor non-functional, but did not impact significantly on carrier health [16], made the receptor an interesting point of research as CCR5 'knockout' humans were readily available for study.

Chemokine receptors are named for their ability to activate chemotaxis upon ligand binding so it is not surprising that CCR5 is linked to diseases where unwanted or excessive migration is a cause. Chronic inflammatory diseases, such as atherosclerosis [17], rheumatoid arthritis [18, 19] and Crohn's disease [20] are linked to CCR5 signalling. These diseases result, in part, from the dysregulation of immune cell migration and activation in the affected tissues. Understanding pathological migration mediated by CCR5 is also important for understanding cancer cell metastasis, where chemokine receptors generally [21, 22] and CCR5 specifically [23, 24], have been shown to be important. For this reason, chemokine receptors including CCR5 have been identified as therapeutic targets for numerous diseases and many attempts have been made to bring chemokine receptor based therapeutics to market [25]. The complete absence of

chemokine receptor antagonist based therapies for chronic inflammatory or metastatic disease at present, however, exemplifies the lack of understanding of how these receptors function. Receptor redundancy, cross-talk, transactivation and the often contradictory signalling events linked to receptor activation suggest that direct blockade of chemokine receptors may not be the most effective route for inhibiting their function. Understanding the intracellular signal transduction events for specific receptors will help to identify therapeutic targets which bypass the plasticity of extracellular receptor activation. The need for novel and specific anti-migratory therapies to treat chronic inflammatory and metastatic disease is great and the blockade of chemokine receptor signalling may only be feasible at the cytoplasmic level. Therefore a greater understanding of signal transduction events triggered by chemokine receptor activation is imperative. CCR5 makes a particularly interesting system to study due to the additional relevance to HIV research and the presence of the CCR5 Δ 32 mutation.

1.2. Chemokine receptors

All chemokine receptors are type A, or rhodopsin like, G-protein coupled receptors and as such share a certain amount of structural and functional homology, as all GPCRs do [26]. Their name derives from their ligands, the chemokines; a portmanteau of chemotactic cytokine. Chemokine receptors are sub-divided into families based on the homology of their ligands: CXC motif chemokines (CXCLs) bind CXC motif or α chemokine receptors (CXCRs). CC motif chemokines (CCL) bind CC motif or β chemokine receptors (CCRs), which together with the CXCRs make the largest two families of chemokine receptors. There is often a high level of ligand sharing within a chemokine receptor family, which is not present between families. CCL3 for example binds CCR1 and CCR5 [27, 28] but shows no affinity for CXCR4, likewise CXCR4 ligand CXCL12 does not interact with CCR1 or 5. Overall sequence homology is not a good indicator of ligand specificity, as some α chemokine receptors have higher homology with β chemokine receptors than other α chemokine receptors. Homology does seem to relate to the way the receptors function upon activation [29], CCR5 and CCR2 which have 79% total sequence homology (UniProt sequence/Blast homology analysis) have some signalling in common which they don't share with CCR1,

which has 56% CCR5 homology [30]. It is these differences in structure which govern the behaviour and signal transduction of different chemokine receptors. Differences in cytoplasmic signalling are governed by factors such as the number of phosphorylation sites the receptor expresses [31], e.g. CCR5 possesses 7 sites whilst CXCR4 possesses 21. Comparisons and contrasts between the structure and function of different chemokine receptors, chemokine receptor families and GPCRs generally is crucial for identifying receptor or family specific signalling. This signalling can then be used to identify therapeutic targets for diseases where specific chemokine receptors are known to be involved.

In contrast to structural similarities and differences that set receptors apart, comparative analysis of chemokine receptor function is often important between receptors, which are structurally different, but appear in the same cells, or are associated with the same biological phenomena. CCR5 and CXCR4, for example, have lower sequence homology, but are both HIV co-receptors. CCR5 and CXCR3 share no agonistic ligands, but are both found in activated peripheral lymphocytes [32]. Interestingly the CXCR3 agonist CXCL11 antagonises CCR5, hinting at possible regulatory mechanisms. Careful analysis of the functional differences between receptors linked to the same biological process or environment may offer insights into the subtleties of specific receptor function. It is now important to understand what is known about chemokine receptor signalling using CCR5 as the model system.

1.3. The G Protein coupled receptor

As their name suggests, all GPCRs couple to G-protein heterotrimers, which become activated upon ligand binding and transduce signals via a wide range of intracellular molecules. The duration and intensity of GPCR signalling is often more important than the fact signalling was triggered, allowing cells utilising GPCRs to process complex but subtle physiological environments and translate signalling into decisive responses such as chemotactic migration. As such activated GPCRs are efficiently desensitised and internalised by recruitment of arrestins after G protein coupled receptor kinases (GRKs) C-terminal phosphorylation, which allows signalling to quickly respond to small changes in the extracellular and intracellular milieu. Activation and desensitisation utilising members of the heterotrimeric G-protein, arrestin and GRK protein families is a cornerstone of GPCR theory and widely held to be a quintessential trait (Figure 1.1.). In the classic model of GPCR signalling activated receptors bind to and activate heterotrimeric G proteins which dissociate into $G\alpha$ and $G\beta\gamma$ subunits that are primarily responsible for signal transduction, which is negatively regulated by arrestin binding. This classical response was later expanded to include arrestin dependent signalling through the formation of signalling protein scaffolds that could lead to specific and independent cellular responses [33-35]. Aside from interacting with heterotrimeric G proteins, GRKs and arrestins, GPCRs have an extensive range of proteins with which they can interact [36], which is why GPCR signalling pathways for any receptor have yet to be fully unravelled. In this respect CCR5 is a typical GPCR with classical signalling through G proteins [27, 37, 38] and arrestins [39] and direct interaction with other proteins described such as JAK2 [40].

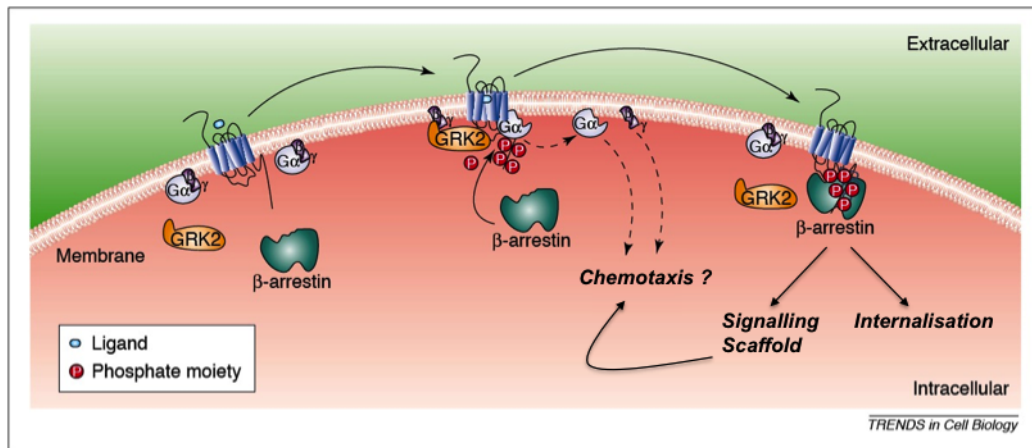


Figure 1.1. Simplified overview of GPCR signalling which represents the most upstream events common to all GPCRs: signalling via heterotrimeric G proteins followed by desensitisation through GRKs and arrestins is as definitive of GPCRs as structural features such as 7 transmembrane domains. G protein and arrestin effectors are numerous and have the ability to feedback into this simple system resulting in diverse functionality. Signalling to chemotaxis can potentially occur via $G\alpha$, $G\beta\gamma$ or arrest scaffold signalling. (Adapted and modified from [41]).

1.4. CCR5 Ligands and receptor activation

CCR5 is known to bind a range of natural and modified chemokine ligands however the effect of ligand binding on receptor activation is not uniform between these ligands. Ligands CCL3, CCL4 and CCL5 are the best characterised CCR5 ligands, the binding of which results in full receptor activation leading to C-terminal phosphorylation and internalisation by clathrin dependent means [15, 27, 42, 43]. Other ligands such as CCL2 and CCL11 can activate some receptor functions but do not result in C-terminal phosphorylation (Figure 1.2.) [42]. A further group of ligands are described as CCR5 antagonists due to their ability to prevent agonist binding without inducing receptor activation; the accuracy of the antagonistic behaviour is in question however due to conflicting reports on their ability to activate CCR5 [27, 42, 44]. Table 1.1. summarises the known CCR5 binding ligands and their effects on the CCR5 receptor.

Table 1.1: CCR5 ligands and binding information. CCR5 C-terminal phosphorylation does not occur upon activation with all ligands.

Ligand	Notes	Refs.
CCL2	Weak agonist. Displaces CCL4 binding with an IC ₅₀ of 0.2 nM.	[5, 42]
CCL3	Higher potency and efficacy than CCR4 and CCR5 in chemotaxis and calcium release assays.	[27]
CCL3 L1	Highest potency and efficacy of the natural CCR5 ligands at inducing internalisation and [³⁵ S]GTPγS binding.	[45]
CCL4	Highest binding affinity for CCR5. Essentially CCR5 specific	[42]
CCL5	Agonist that induces receptor phosphorylation, internalisation, migration and calcium release.	[27]
Met RANTES	Thought to be an antagonist but has shown partial agonism in [³⁵ S]GTPγS binding assays.	[27, 46]
CCL7	Weak agonist: stimulates low levels of receptor activation but does not lead to receptor phosphorylation.	[27, 42]
CCL8	Agonist in migration and internalisation but does not induce CCR5 phosphorylation.	[27, 47]
CCL11	Weak agonist. Displaces CCL4 binding with an IC ₅₀ of 22 nM.	[42]
CCL13	Weak agonist. Can induce internalisation but does not lead to CCR5 phosphorylation. Prevents CCL4 binding with 6 nM IC ₅₀ .	[27, 42]
CXCL11	Antagonist. Displaces CCL4 binding with an IC ₅₀ of 900 nM	[44]

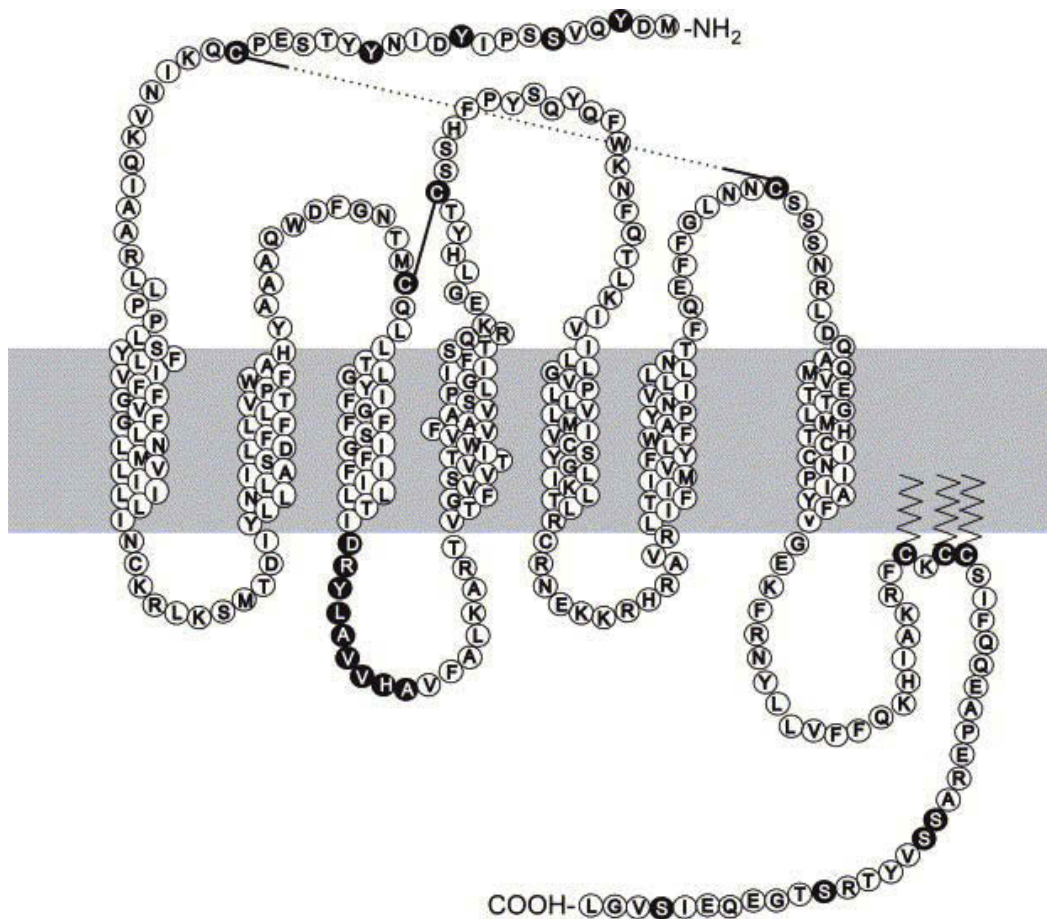


Figure 1.2: The amino acid sequence of CCR5. Important intracellular residues are filled black and represent the 'DRYLAVVHA' motif known for G protein and arrestin binding, sites of C-terminal palmitoylation and the four serine residues required for arrestin binding and internalisation [38].

1.5. The Heterotrimeric G Proteins

Of the three classically associated protein types the heterotrimeric G proteins are the largest group and, accordingly, display a broad range of functions [48]. The G proteins were initially identified as a result of observations made regarding the effects of GPCR activation on adenylyl cyclase activity and cyclic AMP accumulation: some GPCRs, upon activation, increase activity whilst others decrease it [49]. This phenomenon was attributed to stimulatory and inhibitory GTPases termed Gs and Gi, which were shown to be heterotrimeric in nature being comprised

of $G\alpha$, β and γ subunits and display constitutive binding to GPCRs. Ligand induced conformational changes in the GPCR allow the heterotrimeric G-proteins to dissociate from the receptor and the $G\alpha$ subunit to dissociate from the heterotrimer and exchange GDP with GTP to become an active signalling protein. The $G\beta\gamma$ dimer remains at the membrane, but is also free to interact with non-receptor signalling proteins [41].

It became clear as GPCR investigations continued and more associated proteins were purified and identified, that the $G\alpha$ subunit is responsible for the 'classical' signalling functionality of heterotrimeric units as displayed by functional cAMP assays. Eventually sixteen different $G\alpha$ units were identified and the evidence suggested that stimulation and inhibition of adenylyl cyclase was a feature common to only some of these α subunits with others capable of regulating PLC, PI3K or Rho family proteins [50]. Based on their functional similarities $G\alpha$ subunits are sorted into one of four sub-families (Table 1.2), however the nomenclature for these subunits follows a historical precedent and does not allude to this grouping or their function.

Table 1.2: G protein families and α subunits

G Protein family	$G\alpha$ Subunit examples	Signalling function
Gi	αi 1-3, αo , αz	Adenylyl cyclase inhibition
Gq	αq , $\alpha 11$	PLC activation
Gs	αs , αolf	Adenylyl cyclase activation
G12/13	$\alpha 12$, $\alpha 13$	Rho GTPase activation

The most interesting aspect of $G\alpha$ units with regard to GPCR signalling is that a majority of GPCRs can associate with heterotrimeric G proteins containing a variety of $G\alpha$ units. Differential recruitment of these $G\alpha$ subunits may be governed by receptor-ligand binding in a form of biased signalling as seen in some GPCRs [51].

The G $\beta\gamma$ subunit dimer, once disassociated from the alpha units, has signalling capabilities of its own, including inhibition or stimulation of adenylyl cyclase, stimulation of PLC [52], activation of GRK2 and 3 [53] and Pi3K stimulation [54]. These are dependent upon GPCR type and associated G α subunits [48]. Neptune *et al.* demonstrated that G $\beta\gamma$ dissociation from G α_i subunits is essential for chemotaxis to occur downstream of non-chemokine receptor GPCRs such as the dopamine and opioid receptors [55]. There is growing evidence as well, that G $\beta\gamma$ are responsible for signalling to chemotaxis downstream of various chemokine receptors [56-59].

1.6. GRK signalling and CCR5

G Protein Coupled Receptor Kinases (GRKs) represent seven proteins in three families; GRK1 and 7 are found in the retina and form one family, GRK 2 and 3 form one ubiquitously expressed family and GRK 4,5 and 6 form the other [60]. GRKs phosphorylate activated GPCR C-terminal serine residues, which promotes the recruitment of arrestins which, in turn, leads to receptor desensitisation [12]. GRK localisation to the GPCR C-terminus is facilitated by interactions with G $\beta\gamma$ [53] however CCR5 C-terminal phosphorylation can also occur via protein kinase C (PKC) [46]. The role of GRKs as potential signal transducers is however less clear. Although ligand bound CCR5 is a target for GRKs of various type under experimental conditions [12], the significance of this relationship *in vivo* has yet to be investigated, especially, when considering the potential for GRKs to interact with other signalling proteins and mediated arrestin scaffold formation [61]. CCR5 is thought to bind predominantly to GRK2 however [38], which may simplify interpretation of potential signalling pathways associated with the receptor. GRKs are known to interact with other proteins likely to be present at the activated CCR5 receptor that are known players in chemotaxis signalling, such as G α and G $\beta\gamma$ subunits [53]. GRK2 has also been shown to inhibit ERK activation in the CCR2 signalling cascade, a process which is not dependent on CCR2 phosphorylation nor on the GRK G α_q or G $\beta\gamma$ binding domain function. This suggests that GRKs have a protein binding function that works independently of G protein subunits and the receptor itself [62]. It also shows GRKs can form independent complexes with non-receptor proteins, however, their role in CCR5 mediated signalling to migration is unknown.

1.7. Arrestins

Like GRKs, arrestins, the third group of proteins common in GPCR signalling, were initially underestimated in their ability to act as signalling molecules. As more information is discovered about their role in receptor uncoupling and desensitisation, it becomes clear that arrestins can bind a variety of signalling proteins in addition to activated receptors [35, 63]. This suggests that mediation of desensitisation by binding to phosphorylated GPCR C termini and steric hindrance of G α units is just one of their roles. As research has continued, the nature and scope of arrestin dependent signalling has become apparent [64, 65], with the formation and function of arrestin signalling scaffolds dependent to some degree upon factors such as receptor type, associated GRKs and post transcriptional modifications [35, 66]. Signalling scaffolds have no exact definition but generally function to localise signal transduction proteins with one another at a cytosolic location relevant to specific cellular responses [67]. In the case of GPCRs such as the angiotensin receptor 2, ligand activation in the presence of G $\beta\gamma$ binding GRK2 family GRKs leads to classic G protein signalling and internalisation via clathrin dependent mechanism. In this instance, arrestin acts as an adaptor for the relevant endocytotic machinery such as clathrin. Conversely, in the presence of GRK 5 family GRKs arrestin act as a scaffold for signalling proteins such as MEK (MAPKK), which lead to arrestin dependent signalling through ERK and JNK. For both GRK2 and GRK5 family proteins, however, these mechanisms are subject to some degree of crossover i.e. GRK5 is also responsible for recruiting arrestins for internalisation [60]. (Figure 1.3.).

Arrestin dependent signalling has also been shown to extend to activation Src family kinases (SFKs) demonstrating that arrestins are capable of interfacing a wide range of signal transduction proteins with active GPCRs [41]. Understanding the processes governing protein availability for arrestin dependent recruitment to any particular receptor is likely to illuminate a great deal about the receptor signalling capabilities. This fact needs to be considered when investigating the ligand activated signal transduction pathways for CCR5.

Supplementary to ligand-activated processes, it was discovered that arrestins can signal constitutively, adding a new dimension to their role in controlling GPCR induced signalling. Like activation dependent signalling, this constitutive activity varies between receptor types, but is confined to members of the MAPK family; seemingly excluding other non-receptor proteins known to interact with β arrestins upon ligand activation. It is also dependent on localisation of arrestins at the receptor, which indicates that arrestins can bind GPCRs that are not phosphorylated and therefore it does not induce desensitisation/ internalisation pathways in these cases. [68] The relevance of these observations to CCR5 mediated signalling events remain to be clarified.

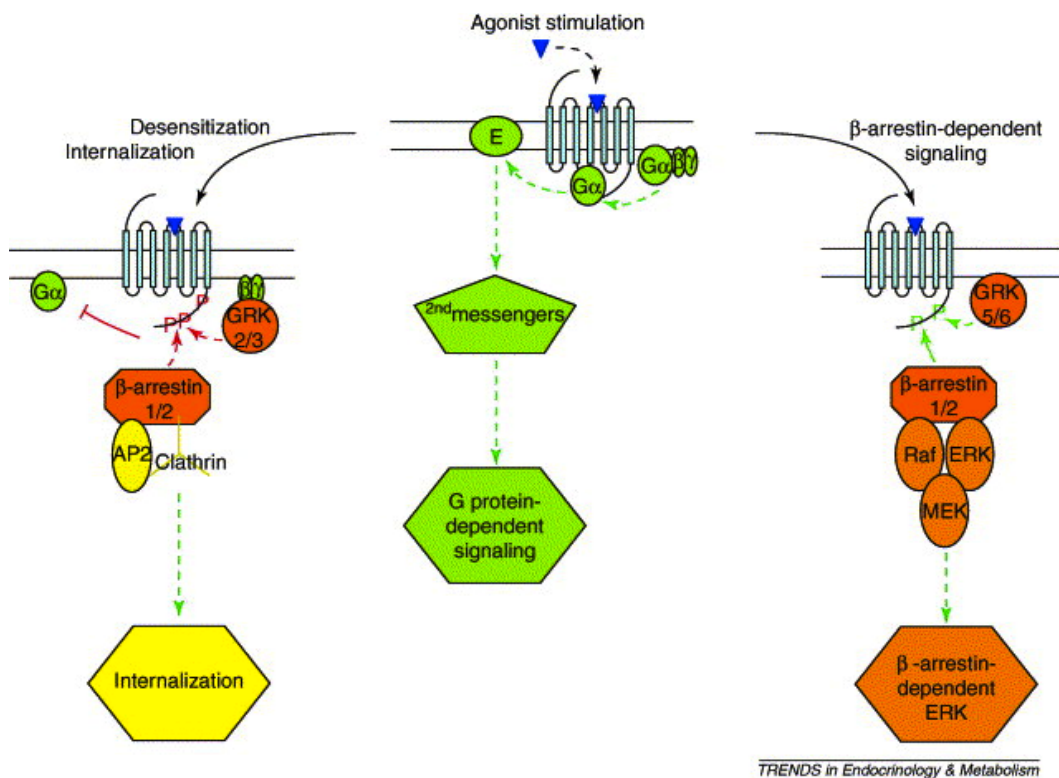


Figure 1.3: Arrestins can act as mediators of endocytosis or signalling scaffolds depending on associated GRKs. Between arrestins, GRKs and G proteins a plethora of signalling is possible upon ligand activation of the receptor through a wide range of molecules: determination of which of these pathways is relevant to CCR5 is crucial to work out intracellular signalling events. (Images from [60]).

A consideration for CCR5 signal transduction determination is the way arrestins have been shown to act in opposition or in synergy with established G α signal transduction pathways for some receptor types, inhibiting or enhancing the magnitude of their effects on downstream targets, which can lead to unexpected results in functional assays [33]. The most important question to address is whether arrestins can function as signalling molecules and scaffolds in a totally independent fashion from G protein activation. There is evidence to support that internalisation and arrestin dependent signalling are indeed independent and that the formation of arrestin dependent signalling scaffolds is controlled by phosphorylation of the GPCR C-terminus and post translation arrestin modification via ubiquitination [66]. Understanding the relevance of arrestin fate in CCR5 mediated signalling to migration will be important but there are other questions that must also be answered such as the role of internalisation in CCR5 mediated migration. Neptune *et al.* demonstrated that the β adrenergic and Angiotensin receptor signalling to migration did not require internalisation but the interaction of these GPCRs with arrestins is likely to be very different due to their much larger third intracellular loops [38].

1.8. Calcium release

Although calcium release is associated with disease in highly active tissue such as the heart [69] and nervous system [70], it is widely used as tool to monitor receptor activated responses. Calcium release occurs quickly upon receptor activation and proportional to the level of signalling [71] which makes monitoring of calcium release incredibly useful for determining the extent of cellular activation in relation to ligand binding and in the presence of inhibitors.

1.8.1. CCR5 signalling to calcium release

Stimulation of calcium release upon activation of CCR5 is well researched and has been shown to occur through G protein dependent means sensitive to both pertussis toxin treatment and modifications to the second intracellular DRY motif which prevent heterotrimeric G protein

binding [72]. Chemokine ligands CCL3, 4 and 5 stimulate CCR5 mediated calcium release in CHO.CCR5 cells [73]. CCR5 ligands that do not result in receptor phosphorylation, however, like CCL8 and CCL13, also induce calcium release [27], which suggests that arrestin dependent signalling is not involved in intracellular calcium fluxes. Cardaba *et al.* have investigated CCR5 mediated calcium release thoroughly and revealed a dependence on lipid raft integrity [74] that may modulate G α isotype specificity as cholesterol depletion results in a reduction in PTX sensitivity without affecting calcium signalling. Signalling to calcium release is believed to occur via G $\beta\gamma$ activation of PLC via PI3K which results in PLC cleaving PIP2 into DAG and IP3, which act as second messengers on calcium channels [52]. Interestingly Cardaba *et al.* were able to demonstrate that calcium release is independent of migration in chemotaxis mediated by CCL3 in THP-1 cells [75]. This suggests that calcium dependent kinases such as Pyk2 and PKC may not be required for migration in this system and could explain the increase in migratory response seen in PKC inhibited cells. Inhibition of PKC might allow an increase of GRK phosphorylation of the CCR5 C-termini, which in turn can increase the GRK or arrestin dependent signalling on which migration is dependent. Despite the apparent independence of CCR5 mediated chemotaxis and calcium, release the calcium flux assay (described by Gryniewicz *et al.* [71]) represents an excellent tool for assessing receptor activation and so may be useful for the clarification of small molecule inhibitor function.

1.9. Chemotaxis

1.9.1. Targeting chemotaxis in chronic immune and metastatic disease

Chemotaxis is the process by which cells migrate towards a gradient of chemical stimulant and is crucial for normal biological processes from embryological growth [76] and development, to wound healing and in immune system function [77, 78]. Unwanted migration of cells is etiological to numerous diverse disease conditions: chronic immune conditions such as asthma, COPD and rheumatoid arthritis are characterised by excessive influx of immune cells which result in physiologic alterations in the affected tissues. Cancer metastasis represents migration

and invasion of malignant tumour cells into healthy tissue and correlates to poor prognosis and is the leading cause of death in hard to diagnose cancers such as lung and prostate cancer. Current therapies for these conditions focus mainly on reducing the effects of this unwanted migration by reducing inflammation or by surgical means. There is a clear need for preventative anti-migratory therapies particularly if they can be targeted against specific cell types or receptor signalling axes. The reason for the paucity in anti-migratory agents is that chemotaxis is not fully characterised and research has produced a picture of multiple and often-conflicting signal transduction to migration. Current anti-metastatic therapies, such as bosutinib [79] doxorubicin [80] and cetuximab do not work on migratory machinery specifically, therefore they have side effects and could not be used to target inflammatory disease. Although chemokine receptors have been validated as targets for anti-migratory therapies for chronic immune disease and cancer [24, 25, 81], the fact that no chemokine receptor antagonist based anti-migratory therapeutics have made it to market, is not through lack of research but because of problems with efficacy [25]. This likely to be due to the fact that chemokine receptor signalling contains redundancy between receptor family types and chemokine ligands often function through more than one receptor. Receptors can also signal horizontally through dimerised [82, 83] and arrayed species [84] and transactivation between receptor types can occur [85].

1.9.2. The cytoplasmic mechanism of chemotaxis

Chemotaxis has been shown to be mediated by GPCRs and receptor tyrosine kinases such as cytokine receptors. GPCRs such as β 2-adrenergic and M3-muscarinic receptors are known to signal to chemotaxis [55] but it is the chemokine receptors, as their name suggests, which are most well-known for signalling to migration. The specifics of signal transduction, which are discussed below, are complex and have receptor and cell specific components which make generalised signal transduction to migration difficult to describe. There are events that occur in chemotaxis that are considered to be conserved however; migration is a process driven by interaction with the extracellular matrix, polarisation and cytoskeletal rearrangements (Figure 1.4.).

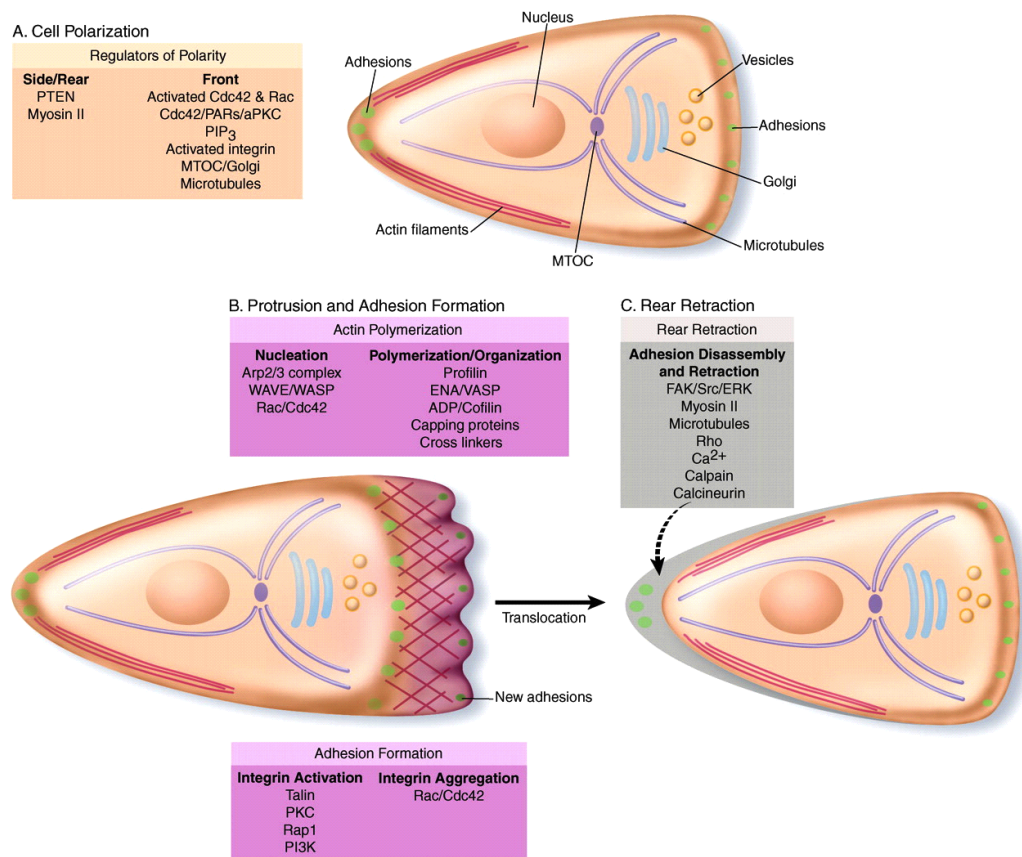


Figure 1.4: The general processes and signalling molecules thought to be involved chemotaxis. This overview does not take into account cell or receptor specific signalling events or explain how monocytes, which do not require PI3K for polarisation, function. Image from [76].

Polarisation allows the cell to determine the spatial orientation of an extracellular stimulant concentration gradient by producing intracellular gradients of signalling molecules [76]. The cytoskeleton is re arranged to allow movements towards this gradient by interaction of the cytoskeleton with motor proteins and this force is transmitted through adhesion present in membrane structures such as filopodia [86] and podosomes [87-89]. Disassembly of this machinery at the trailing edge of cells is also important and this is again controlled by polarisation. Membrane structures such as podosomes [90] and invadopodia [91] also facilitate the squeezing of cells into other tissue. Signalling to chemotaxis must involve a concerted effort from numerous and diverse signalling proteins in multiple cascades from polarising PI3Ks to regulatory Rho GTPases and cytoskeletal modulators such as cortactin, dynamin and nWASP.

Identification of CCR5 receptor specific signalling events in chemotaxis will allow pathological migration to be targeted without the problems associated chemokine receptor redundancy or interference with signalling in non-diseases tissue. In order to resolve CCR5 specific signal transduction events the current understanding of receptor signalling to cellular responses such as migration and calcium release will be reviewed.

1.9.3. Chemokine receptors and chemotaxis

As described, chemokine receptors are well known for their ability to signal to chemotaxis upon activation by their chemokine ligands. Signalling to chemotaxis does not appear to be uniform between receptor types however. $G\alpha$ protein isotype specificity explains only some differences between receptor signalling as chemokine receptor signalling to chemotaxis also occurs through $G\beta\gamma$ and β arrestin dependent pathways [34, 39, 55, 92]. CXCR4 signalling to migration has been shown to occur through a $G\beta\gamma$ -PI3k-ERK1/2 dependent pathway [58, 59, 82]; CCL3 stimulated CCR1 mediated migration has been shown to be sensitive to pertussis toxin (PTX), treatment and PLC and p38 MAPK inhibition [51]. CCL3 stimulated CCR5 mediated migration has also been shown to be PTX sensitive [74, 93] but chemotaxis signalling by the receptor also occurs through arrestin dependent signalling

1.9.4. CCR5 mediated G protein signalling molecules associated with chemotaxis

G $\beta\gamma$ subunits had been shown to be effectors of PI3K, the activation of which is known to be a crucial mediator in chemotactic response [94]. In addition, G $\beta\gamma$ subunits directly affect PLC, up-regulating second messenger production, and Ras2 exchange factors that can also be directly activated by second messengers suggesting signalling feedback in association with G protein activation [57]. Small molecule inhibition of PI3k in neutrophils, highly chemotactic leukocytes, show a marked reduction in GPCR mediated chemotaxis [95]. This indicates $\beta\gamma$ subunits may operate in a similar way down stream of activated CCR5. This may suggest an interaction between G $\beta\gamma$ and the PI3K containing arrestin signalling scaffolds later shown to exist by Cheung *et al.* however this interaction remains unresolved. The evidence to suggest that CCR5 signals mainly through G α_i [38] and to a lesser extent G α_q [40] is strong, however, there is still the possibility of G $\alpha_{12/13}$ interaction as it has not been categorically disproven. If G protein signalling is governed by the relative concentrations of G α units present in the cytosol, then G $\alpha_{12/13}$ signalling may have been overlooked if signalling is occurring at a lower levels, or can be preferentially selected by ligand biased signalling. Figure 1.4. demonstrates the breadth of possible G α signalling to chemotaxis [96], however the relevance of investigating non-G α_i G proteins in CCR5 mediated migration may be irrelevant as it has been shown to be completely abrogated by pertussis toxin treatment [74, 93]. Pertussis toxin is a well-known inhibitor of G α_i dependent signalling, but its use raises some questions as to the ability of PTX treated G α_i receptors to signal normally through G $\beta\gamma$ and arrestins if G α_i dissociation cannot occur. The observations made by Cheung *et al.* [39] (see below) describing arrestin scaffold dependent signalling to migration from CCR5 are certainly not explained if signalling to migration occurs wholly via G α_i dependent signal transduction.

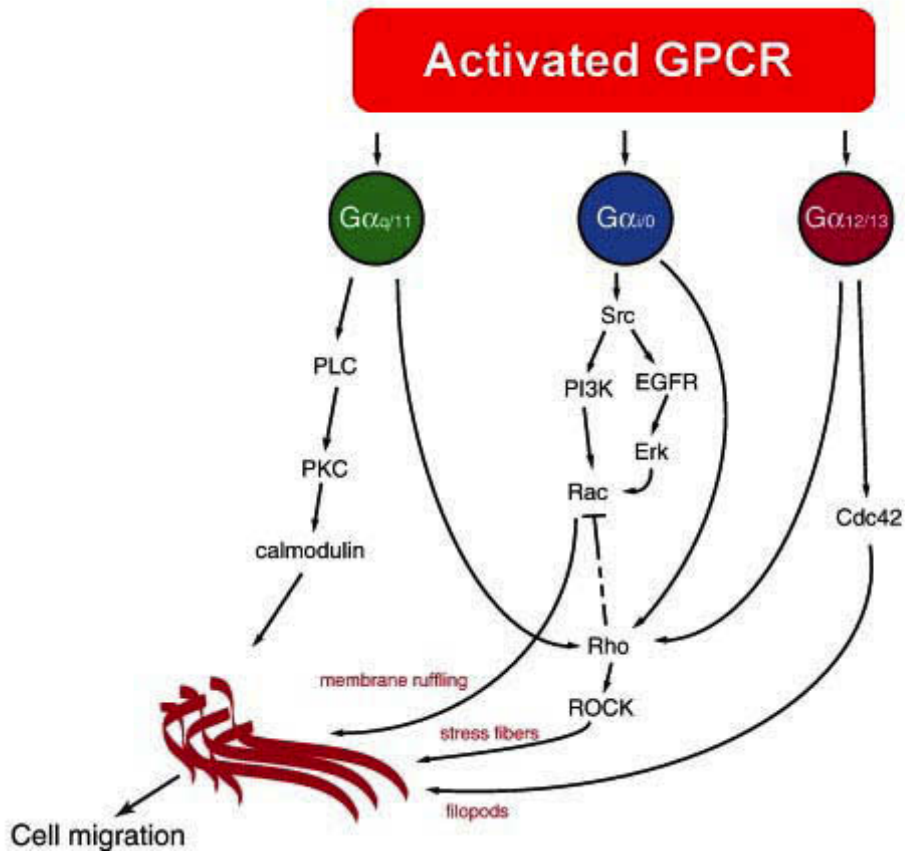


Figure 1.5: Signal transduction pathways leading to migration in receptors signalling through different G proteins. Rho and ROCK form a common signalling pathway making them possible targets for SiRNA silencing. (Image from [96]).

1.9.5. Arrestins and GRKs in CCR5 signalling

GRKs and β arrestin are known to be involved in CCR5 phosphorylation and desensitisation. Oppermann *et al.* were able to show that phosphorylation on four specific serines in the C-terminus of CCR5 by GRKs is required for arrestin recruitment and internalisation [46]. Over expression of both GRK2 and GRK5 family GRKs increases CCR5 phosphorylation, however, knock down of GRK2 and GRK3 reduces phosphorylation markedly, although not completely. Additional inhibition of PKC further reduces CCR5 phosphorylation, showing that the arrestin recruitment is controlled primarily by GRK2/3 and PKC mediated phosphorylation. The group

expanded this work and provided additional evidence that GRK 2 and 3 and PKC are responsible for phosphorylation and desensitisation of CCR5 under normal cellular conditions [61]. Interestingly, it was shown that isolated β arrestins are able to bind to phosphorylated and non-phosphorylated CCR5 C-terminal derived peptides with equal affinity. This suggests that β arrestin has numerous domains capable of CCR5 binding, which might allow allosteric modification of arrestin when involved in a signalling complex with the receptor in either active or inactive forms [43]. The DRYLAVVHA motif (Figure 1.2.), which is highly conserved in many chemokine receptors and has been shown to be involved in heterotrimeric G protein binding [13] proves to be the second β arrestin binding site. Synthetic peptides representing CCR5 mutants, where DRY residues were replaced with inactive alanines, show no binding affinity towards β arrestin compared with peptides representative of WT CCR5. Despite a lack of relative conformation, the use of synthetic peptides provides strong evidence for the interaction between β arrestin and the DRY motif and shows how desensitisation occurs by arrestin mediated inhibition of heterotrimeric G protein binding at the second intracellular loop. Arrestin binding to the DRY motif has been shown by Lagane *et al.* to be a prerequisite for chemotaxis and the fact that arrestin can bind to non-activated receptors [97] might suggest a role for the protein in constitutive basal migration.

Arrestin interaction with CCR5 is not required for ERK MAPK phosphorylation, with levels actually increasing for some truncated receptor species [98]. As calcium levels also increase due to a lack of desensitisation, this leads to the theory, that calcium activated kinases such as Pyk2 might be responsible for the increases in ERK phosphorylation. Pyk2 can interact with numerous signalling molecules (Figure 1.6). Pyk2 appears to represent an interesting target for investigation in relation to CCR5 mediated chemotaxis however the independence of migration and calcium release [75] may indicate that Pyk2 function outside of the scaffold may not be important.

A relationship between Src family kinases (SFKs), such as Lyn and the activation of ERK1/2 MAPKs downstream of CCR5 was described by Tomkowicz *et al.* who showed that CCR5 mediated ERK1/2 phosphorylation occurred via a nonspecific Src kinases inhibitor (PP2) sensitive pathway [99]. PP2 inhibits numerous Src kinases such as Lyn and Hck, so to determine which of these is activated by CCR5, specific inhibitory peptides for Lyn and Hck were produced and used to treat CCR5 expressing macrophages. The Lyn specific protein alone was shown to have a significant inhibitory effect on CCL4 stimulated migration [99]. This shows that the SFK Lyn operates downstream of CCR5 and upstream of ERK1/2 MAPKs in macrophages. Whether this relationship is receptor or cell type specific was not addressed by the group, however. This provides an interesting conundrum, because ERK and c-Src activation is associated with signalling to migration in many receptor systems [39, 100-102]. The mechanism of CCR5 signalling through SFKs to chemotaxis was expanded by Cheung *et al.* who demonstrated that arrestin acted as a scaffold protein for the formation of a Lyn/Pyk2/PI3K complex necessary for chemotaxis in macrophages [39]. Phosphorylation of Lyn, Pyk2 and PI3K upon ligand binding to CCR5 was demonstrated, however the fact that inhibition of any of these proteins by small molecules or silencing with siRNA resulted in a significant decrease in chemotaxis was puzzling. This led Cheung *et al.* to investigate the possibility of a physical association between the three proteins around which their functionality might be based [39].

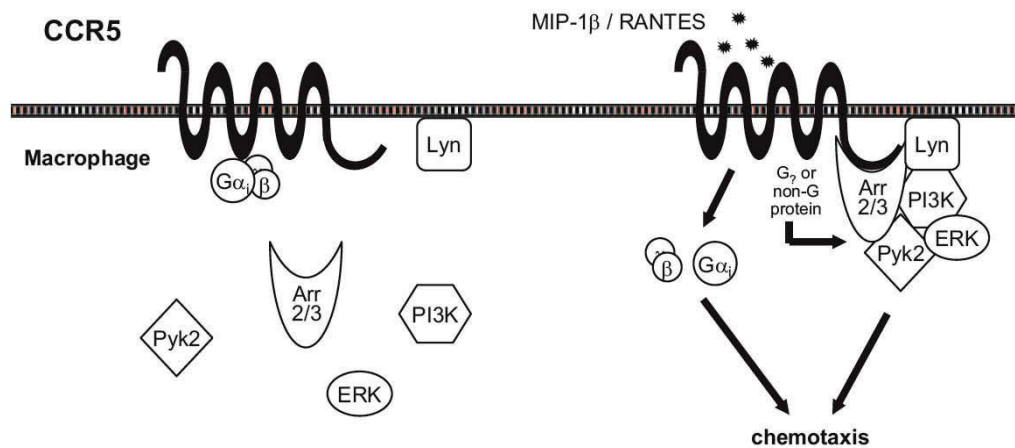


Figure 1.6: The arrestin scaffold proposed by Cheung *et al.*: If any one of the components are missing, with the exception of ERK, chemotaxis is reduced. The role of GRKs and G proteins with this scaffold remains to be seen. (Image from [39]).

An arrestin-Lyn-PI3K-Pyk2 complex was identified by co-precipitation; further investigation into the relationship between arrestins and these binding partners showed a significant level of constitutive association between all four components which not only considerably increased upon ligand activation but promoted the additional co-localisation of ERK. This is strong evidence that arrestins are capable of promoting signalling events not related to desensitisation or internalisation of CCR5. Signalling scaffolds of this nature have been shown to occur in association with GRK5 family GRKs [41]. Investigations into whether GRK5 associates with CCR5 to allow the formation of such complexes or whether arrestin are divided between internalisation and scaffold roles is an important question that needs to be answered. In particular the differential phosphorylation of the CCR5 C-terminal by GRK and PKC species in relation to arrestin recruitment may reveal novel signalling to migration.

It has been shown that CCR2, a chemokine which has a 79% total sequence homology and 80% homology in the amino acid residues of the 4th intracellular loop can bind to the protein FROUNT in this highly homologous area [103]. Suppression or over expression of FROUNT correlates to a decrease or increase in chemotaxis, respectively [30]. FROUNT can also regulate chemotaxis in CCR5- occupying the signal transduction location immediately downstream of the

receptor and immediately upstream of PI3K; [29] the previous most upstream transduction protein known for CCR5 mediated chemotaxis. [103] How FROUNT interacts with other PI3K effectors such as $\beta\gamma$ subunits and β arrestin scaffold has yet to be determined.

Over all it is clear that the role of arrestin in endocytosis and signalling is far from clear with regards to CCR5 mediated migration. The possible interactions between arrestins and other proteins in the cytoplasmic milieu is only beginning to be resolved and may reveal novel signal transduction to migration which could be therapeutically targeted.

1.10. PI3Ks: linking $G\beta\gamma$ and arrestins to chemotaxis

Phosphoinositide-3-Kinases (PI3Ks) are ubiquitously expressed kinases that phosphorylate membrane bound phosphoinositides (PI). Phosphorylation of PI allows targeting of pleckstrin homology domain containing effectors, such as PKB (Akt), phospholipase C [104] and dynamin 2 [105-107], to the cell membrane. PI3Ks work antagonistically with lipophosphatases such as PTEN and SHIP to maintain polarisation [78]. This makes PI3K central to migration, where cell polarisation is facilitated by the interplay of PI phosphorylation by PI3K [76, 108] and phosphatase action. PI3Ks are associated with other cellular processes such as proliferation and protein expression [78] and are of particular interest to cancer biologist because of their propensity to be over expressed in cancers cells [109]. PI3K has several isotypes, with PI3K γ being associated with GPCR signalling through $G\beta\gamma$ [54, 110, 111] and the α , β and δ isotypes with signalling via receptor tyrosine kinases (RTKs) [112, 113]. There is some evidence that the β isotype can be activated by some GPCRs [114]. It is therefore likely that CCR5 mediated signalling occurs through the γ isotype. PI3K has been linked to CCR5 activation as part of the arrestin dependent signalling scaffold describe by Cheung et al. [39] (see above), but $G\beta\gamma$ released from activated CCR5 should also be able to activate the protein and there is also the possibility that $G\alpha_i$ could lead to PI3K signalling, too [96]. Aramori *et al.*, however, failed to detect phosphatidylinositol hydrolysis upon CCR5 activation by CCL3, 4 and 5, presumably by

lack of PLC recruitment by PI3K activity [12]. This represents the kind of contradictory evidence that complicates the understanding of signalling to migration. This is further complicated by the report of Volpe *et al.* that monocytes do not require polarisation for migration like larger adherent cells [115]. How arrestin signalling scaffolds, which require PI3K for their formation [39], fit into these observations remains to be clarified.

What is clear is that clarification of PI3K function downstream of CCR5 will be important in understanding how chemotaxis is mediated by the receptor. Small molecule inhibitors such as LY294002 [116] and wortmannin [117] have been widely used to probe PI3K function in other signalling systems and these, along with recently described PI3K γ specific inhibitors [118], should allow the role of PI3K in CCR5 mediated chemotaxis to be better described without resorting to costly proteomics.

1.11. CCR5 and JAK/STAT signalling

In normal tissue the JAK/STAT signalling axis is best known for its role in up regulation of protein transcription stimulated by cytokine receptors [119]. JAK/STAT mediated up regulation of protein transcription has been linked to migration and increased JAK/STAT activity is a marker in some cancer cells, where metastasis occurs [120, 121]. JAK/STAT signalling may be relevant to CCR5 mediated chemotaxis because cytokine receptor transactivated GPCRs are thought to link through signalling scaffolds that may contain JAK/STAT proteins [85]; and more importantly JAK2 has been shown to interact directly with CCR5 [40, 122]. The interaction with chemokine receptors has been described by several groups [122] and it has been shown that chemokine receptor activation leads to JAK-STAT phosphorylation in a similar fashion to cytokine receptors, which presumably leads to increases in protein transcription in a similar way. Additionally, JAK/STAT and PI3K signalling has been linked to cytokine mediated migration and invasion of hepatocellular carcinoma cells [123]. What is not known is whether the JAK2 interaction with CCR5 has any cytoplasmic signalling role with regard to chemotaxis. JAK/STAT are associated with migration through other receptors [124] and have been linked to calcium release

downstream of chemokine receptors [125]. Understanding the role, if any, of JAK2 and STAT in migration will be important in characterising CCR5 function and the small molecule tools to investigate the idea are readily available. Chemically diverse species such as cucurbitacin I [126], JAK2 inhibitor 2 [127] and STAT3 inhibitors III and VIII [128] have all been shown to reduce STAT3 phosphorylation. These inhibitors will make useful tools to investigate the exact nature of JAK2-CCR5 interactions in relation to chemotaxis signalling.

1.12. Dynamin

Dynamin is a 100 kD protein, that was first identified in 1989, when it was isolated from microtubules with which it was bound: the interaction with microtubules was initially linked to ATPase activity [129]. Later on it was shown that dynamin is, in fact, a GTPase [130] and that there are several types: dynamin I is primarily found in neurones where it is involved in synaptic vesicle endocytosis [131, 132] and it has been linked with several neurological processes such as long-term memory formation [133]. Dynamin 2 is ubiquitously expressed and is found in all cell types, dynamin 3 is primarily found in the testis. Dynamin 2 interacts with numerous GPCRs as well as non-GPCR receptors, including the chemokine receptor CCR5 [134] and various cytokine receptors [135]. Dynamin 2 is of particular interest, firstly because of its ubiquitous expression, and secondly because of the range of proteins and functions dynamin has been associated with, which are directly relevant to CCR5 mediated chemotaxis. Dynamin 2 has been shown to be involved with internalisation [136], during which it associates with clathrin coated pits [137] and arrestins [64], but it also has direct links to migration [77]. The range of dynamin interacting proteins, which have also been linked to migration is great and offers many potential routes of signal transduction from CCR5 to mediate dynamin function (see below).

1.12.1 Dynamin in disease

Mutations in the dynamin 2 gene have been shown to be causal in Charcot–Marie-Tooth disease, [138], peripheral neuropathy and centronuclear myopathy [139]. Dynamin 2 has been

identified as a susceptibility gene for late onset Alzheimer's disease [140]. It is not surprising, due to the role of dynamin in synaptic vesicle formation, that mutations in dynamin also affect psychological function and have been associated with alcohol tolerance in drosophila models [141].

More relevant to this thesis is the function of dynamin in diseases where internalisation and migration are causal. Inhibition of dynamin has been shown to block botulin neurotoxin uptake [142] but this process has been shown to be both clathrin dependent [143] and independent [144], suggesting that caveolin dependent endocytosis is also dependent on dynamin. Similar discrepancies are observed with HIV internalisation. Carter *et al.* demonstrated that dynamin and clathrin are required for CCR5 mediated viral entry in macrophages [145], but CCR5 mediated viral entry in trophoblasts is independent of dynamin [146]. Differences in cytoplasmic proteins between cell types may explain these observations, but there is also evidence that different HIV strains can recruit endocytosis machinery differentially, with some strains internalising through dynamin dependent means and others not [147]. The differential recruitment of endocytosis machinery by activated CCR5 receptors may be important in explaining cell specific signalling to migration.

Dynamin 2 has been linked to migration dependent pathologies such as the growth and malignancy in several cancer types such as breast cancer [148], pancreatic ductal carcinoma [149] and in immortalised cancer cell lines [150]. The role of dynamin in migration extends to non-cancerous cells (reviewed in [77]), which means that it may be a potential target in immunological disorders where unwanted or excessive migration of immune cells causes the disease, such as asthma, COPD and rheumatoid arthritis, where CCR5 receptor activation is also thought to be involved.

1.12.2. Dynamin structure

Dynamin is a 100 kD proteins that consists of a GTPase domain, a middle or stalk domain, a pleckstrin homology domain, a GTPase effector domain (GEF) and a low order pleckstrin homology domain [151, 152]. Resolution of the crystal structure of dynamin without the low order PRD demonstrated that the protein folds back along its self at the PH domain so that the GTPase and PRD would be in close proximity [153, 154] (Figure 1.7.).

The domains of dynamin have been linked with various protein-protein interactions and processes: The GTPase domain is the site of GTPase turnover and is associated with changes in conformation which result in the motor action of dynamin [155, 156]. Mutation of the GTPase domain results in the dynamin K44A dominant negative mutant, which has been used to investigate the role of dynamin GTPase function in both endocytosis [157] and migration assays [158]. The GTPase effector domain has been shown to regulate self-assembly in a Src phosphorylation dependent fashion [159], indicating that regulation by cytosolic kinases can promote dynamin function. The pleckstrin homology domain of dynamin is associated with the targeting of dynamin to phospholipids, such as PI3K product PIP₂, at the membrane [105, 106] a process that has been shown to be a prerequisite for phagocytosis [160, 161]. The middle domain of dynamin has been shown to be the site of self-assembly [105, 162], but also to be the site of direct microtubule [163] and actin binding [164]. These interactions enhance dynamin GTPase function and promote assembly into polymerised dynamin rings. The most interesting dynamin domain with regard to CCR5 mediated migration is the proline rich domain (PRD). The PRD facilitates binding of dynamin to SH3 domain containing proteins such as Grb2 [165] but also with other adaptor proteins [100] and directly with microtubules [166]. Interaction with proteins at the PRD can increase dynamin GTPase activity [167] and promote self-assembly. Phosphorylation of the PRD by the MAP Kinase ERK can also down regulate microtubule binding whilst increasing GTPase activity [168] which suggests that the regulatory effects of the PRD on dynamin function are manifold. Binding of adaptor proteins such as Grb2 to the PRD has also

been shown to target dynamin to phosphorylated tyrosine residues, presumably through Grb2 SH2 domains, which may explain how dynamin function is directed in the cytoplasm.

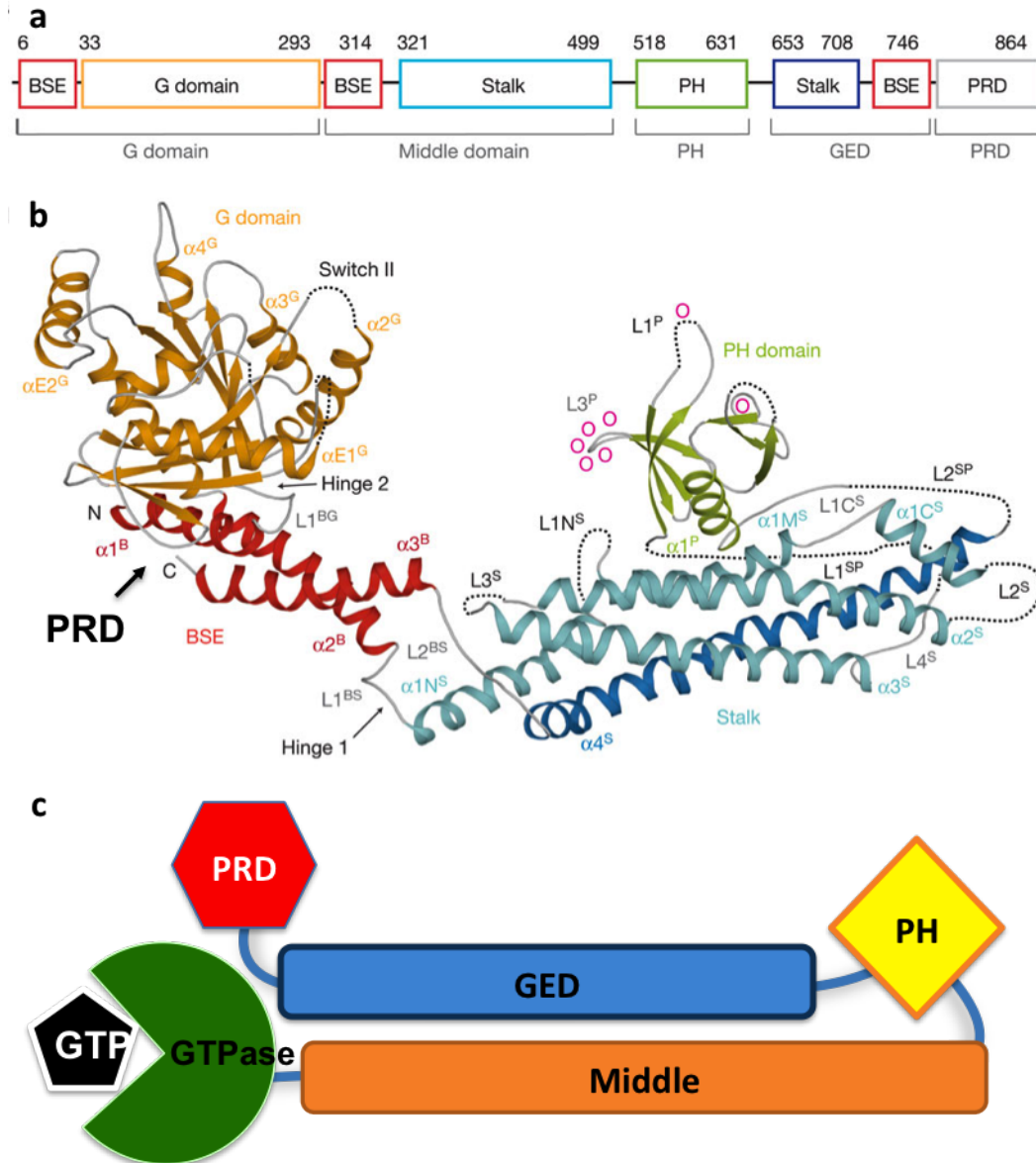


Figure 1.7: The structure of Dynamin. (a) An overview of the dynamin 1 AA sequence and its relationship to the functional domains of the protein (stalk = middle) (Bundled signalling element (BSE) = GTPase effector domain (GED)). (b) The solved Δ PRD dynamin crystal structure demonstrating the spatial relationship between domains. (c) Cartoon representation of the 3D structure of dynamin representing the spatial relationship between the domains. (a&b) image from [153].

1.12.3. Endocytosis

Dynamins are primarily known for their role in clathrin coated pit dependent endocytosis, considered its classic mode of action [157], however, not all endocytosis is dependent on dynamin. Acetylcholine receptors, for example, have been shown to internalise via a cholesterol dependent, dynamin independent mechanism in CHO and C2C12 muscle cells [169]. Also dynamin 2 is not required for massive endocytosis (MEND), a process where up to 75% of the plasma membrane can be reversibly endocytosed. MEND is also independent of actin cytoskeletal rearrangement and other mediators of classic endocytosis and thought to be dependent on calcium flux. [170] Dynamin is also involved with endocytosis in a CCP independent fashion: clathrin independent dynamin 2 dependent endocytosis that also requires Rho is utilised by the IL-2 receptor and in the endocytosis of Clostridium toxin. This shows that dynamin can recruit a range of proteins to facilitate internalisation, which may be dependent on receptor mediated signalling or the available cytoplasmic protein complement [143]. 'Typical' endocytosis involving clathrin coated pit formation and vesicle stabilisation and scission by dynamin is well described with numerous groups proposing models for the exact mechanism of the process [171-176]. The precise details of dynamin vesicle scission are not relevant here, but there are important points about the process which should be mentioned. Firstly, endocytosis requires dynamin in two individual steps; for the formation of stabilised 'U' shaped pits and later for the pinching of 'O' shaped vesicles [177]. Secondly, dynamin polymerisation into a ring is required for endocytosis with the pinching action dependent on GTPase hydrolysis conveying conformational changes to the ring macro structure. Dynamin rapidly disassembles post vesicle scission [105]. Finally, the most recent models based on crystallographic structures have proposed that the vesicle scission function of dynamin can occur without the PRD domain attached, which suggests that the PRD may be more important for the targeting of dynamin or for other protein interactions with the molecule [155].

Dynamin dependent endocytosis is directly relevant to CCR5 function: the dynamin 2 dominant negative mutant K44A has been used to show that CCR5 internalisation is dependent on

dynamin 2 [12] and that dynamin dependent internalisation occurs in THP-1 cells [134, 178] which raises the possibility that other dynamin dependent signalling may originate from this interaction. This is particularly interesting as other GPCR signalling proteins present at the activated CCR5 receptor are known to interact with dynamin such as arrestin 2 [64] and G β γ [107] which themselves have been linked to chemotaxis [39, 57].

1.12.4 Dynamin and cell migration

As more is discovered about dynamin interactions and its cellular localisation it becomes more apparent that it is also involved in cell migration (reviewed in [77]). The work of McNiven *et al.* demonstrated that in addition to functioning as a scission mediator during endocytosis, dynamin formed interactions with numerous actin binding and cytoskeletal modelling proteins [179], such as nWASp, Arp2/3 and links to actin via PRD interaction with Abp1. Binding of dynamin to the actin binding protein cortactin was demonstrated to be a requisite for morphological changes in fibroblast which occur during migration and spreading [180]. This is interesting because cortactin, like dynamin binds to Grb2 which may indicate cross linking of these proteins. Even more interesting is the fact that c-Src mediated phosphorylation of cortactin is facilitated by Grb2 [181], which suggests that targeting of Grb2 SH2 domains via c-Src phosphorylation targets may be central to dynamin cortactin interaction and function and therefore ultimately migration. These observations were made in fibroblasts, so cell-type dependent factors, such as phosphoinositide based polarisation need to be validated. Dynamin appears to link the actin cytoskeleton to the membrane, however, dynamin is not just acting as an anchor. The dynamin-cortactin interaction governs actin filament organisation in membrane structure [182, 183], an observation which was later validated with the use of small molecule dynamin inhibitors [184]. The role of dynamin in the organisation of actin fibres is not fully characterised, however there is experimental evidence which may go some way to describe this function. Dynamin has been shown to be crucial in the uncapping of the actin regulatory protein gelsolin from the barbed end of actin fibres [164]. Actin polymerisation occurs predominantly at the barbed end with the disassembly occurring at the pointed end [185]. This data indicates that

dynamin may directly mediate actin dynamics as a complex with gelsolin, however, gelsolin function is also known to be calcium dependent [186], which makes this interaction contradictory to CCR5 mediated chemotaxis in THP-1, which has been shown to be independent of calcium [75]. The interaction of dynamin with actin is not limited to binding through PRD associated actin binding proteins such as gelsolin and Abp1/2; Gu *et al.* demonstrated that PRD deficient dynamin mutants not only bind to actin but are able to facilitate actin polymerisation. This suggests that the function of the PRD is primarily to target dynamin within the cytoplasm. The effect of PRD truncation on chemotaxis was not determined, so the effect of decreased dynamin targeting on migration has not been described. This effect may also be cell type specific. Knock down studies with dynamin have shown that actin polymerisation can still occur and mice lacking dynamin show increases in the number of focal adhesions in fibroblasts [187]. These observations suggest, that opposed to acting as a primary instigator of actin polymerisation, dynamin regulates and directs actin polymerisation alongside other proteins. The evidence for the role of dynamin in migration extends into disassembly of focal adhesions, which has shown also to be dependent on clathrin which indicates endocytosis may be involved in this process [188]. The list of potential proteins through which dynamin may regulate actin dynamics is increasing continually [189] but interactions with cortactin [190], Rho [191] and ROCK [192] are obvious places to start and are likely to fit into CCR5 mediated responses.

There is a considerable evidence for a role for dynamin in conjunction with non-receptor tyrosine kinases and SH2/SH3 domain containing adaptor proteins in the formation and stabilisation of membrane protrusions such as podosomes and filopodia [87-90, 100, 135, 183, 193] which are important for migration and invasion [76] and may offer more potential targets for therapeutic intervention.

1.12.5. Grb2 and chemotaxis

As stated above, Grb2 is known to interact with dynamin via the PRD, where it can increase basal GTPase activity and also target dynamin to phosphorylated tyrosine residues. There is also

a link between Grb2 and migration that may or may not be linked with dynamin, and is certainly an interesting area for investigation. Canonical Grb2 function involves receptor tyrosine kinase activation leading to Ras, Sos and Grb2 signalling to PI3K [112] which leads to RTK mediated migration [135]. Rationally designed Grb2 inhibitors have successfully targeted RTK signalling to migration [112, 113] and siRNA knockdown of Grb2 also results in a reduction in migration in this system. Interestingly Grb2 has been shown to associate with dynamin through guanine nucleotide exchange factors (GEFS) in the RTK signalling system. The association between Grb2, dynamin and migration occurs elsewhere. Grb2 is thought to link dynamin to Src tyrosine kinase sensitive adapter protein complexes, which are required for actin polymerisation in podosomes. Interestingly, Δ PRD dynamin did not prevent actin polymerisation in this system, which indicates, that the Grb2 PRD interaction is more important in the targeting of dynamin than in enhancing its function. Gilleron *et al.* demonstrated that c-Src inhibition reduces dynamin targeting to the membrane, which might be explained by Grb2 interactions [194]. The association of dynamin and Grb2 with membrane protrusions such as podosomes has been shown in other research [88, 89, 195], which may suggest that CCR5 signalling to such structures may also involve these proteins. There is evidence to suggest that GPCRs can signal to Grb2 signalling: CCR1 homologous virally encoded chemokine receptor US28 activation by CCL5 confers pertussis toxin sensitive signal transduction which results in the association of Grb2 and focal adhesion kinases in fibroblasts [134]. This interaction is required for migration in US28 transfected smooth muscle cells, however, this also raises questions regarding receptor/ cell type specificity described above. If polarisation via PI3K activation is not required for chemotaxis in monocytes, other signal transduction processes cannot be assumed to be conserved either. There is no literature linking CCR5 signal transduction through Grb2 directly to migration, however, there are reports of Grb2 being involved in phosphatase regulation in CCR5 mediated macrophage chemotaxis stimulated by CCL4 [101] and even suggestions that Grb2 might interact directly with CCR5 C-terminal tyrosine 339 [196]. Considering the links to chemotaxis, dynamin function and CCR5, Grb2 represents an interesting point of investigation in relation to the signalling events that occur upon receptor action and may offer novel insights into these

events. Small molecule inhibitors for Grb2 SH2 domains do exist but are not commercially available.

1.12.6. Other cytosolic interactions of dynamin

Outside of dynamin's interaction with cytoskeletal chemotaxis mediators, there are numerous other protein interactions that may link dynamin to the CCR5 signalling cascade. The interaction between PIP₂ via its PH domain has been discussed, however, this interaction may extend to a more intimate relationship with PI3Ks. For example, dynamin 2 is required for the formation of phagosomes and PI3K is required for dynamin recruitment to the phagosome. This suggests that PI3K operates upstream of dynamin 2 in this interaction [160]. Gridin, a ubiquitously expressed mammalian actin binding protein, accumulates at the leading edges of polarised cells and also interacts with PKB and activated dynamin 2 [197], suggesting that this protein might scaffold the well know PI3K target to the site of phosphoinositide phosphorylation. The idea that dynamin targets PIP₂ effectors is expanded by the fact that dynamin 2 PRD interacts with PLC SH3 domains [180, 198], which suggests, that dynamin may facilitate calcium release by second messenger generation. Calcium sensitive PKC phosphorylate dynamin PH domains, which increases GTPase activity and interaction with Gβγ [199] and therefore the possibility for self-regulation or GPCR dependent function is present. The possibility for GPCR dependent regulation of dynamin is cemented by the fact that arrestins target GPCRs to clathrin coated vesicles [136], which potentially put arrestins, Gβγ subunits and dynamin at the same locus upon ligand activation of receptors. The complexity of dynamin signalling is redoubled when considering the evidence that links dynamin to MAPK signalling. Dynamin 2 is required for MAPK activation in COS-7 cells, where overexpression of dynamin K44A prevents activated MEK from accumulating in the cytosol. This suggests that dynamin mediated endocytosis of activated MEKs is required for phosphorylation of MAP kinases [200]. CCR2 activation also leads to dynamin dependent phosphorylation of ERK in THP-1 cells [178]. ERK phosphorylates dynamin 2 at the PRD domain which results in a decreased GTPase activity and microtubule binding

affinity, which opens up another potential self-regulatory signalling system relevant to CCR5 signalling [168].

1.12.7. Small molecule inhibition of dynamin

Inhibition of dynamin function is necessary to allow its function in dynamic cellular responses to be determined. Prior to the discovery of small molecule inhibitors of dynamin overexpression of dominant negative or truncated mutants or knock down with siRNA were the only realistic methods to modulate dynamin in cells. These methods, whilst successful, have distinct disadvantages, such as the long time frame of transfection and the altered cytoplasmic concentrations of protein, which in turn may alter signalling. The need for small molecule inhibitors of dynamin, which would allow quick and reversible modulation of the protein function, was therefore great. The first dynamin inhibitor, dynasore, was identified by Macia *et al.* from a screen of over 16,000 compounds and it reversibly binds dynamin and prevents internalisation [158]. Soon after, numerous other dynamin inhibitors were discovered by other groups (Table 1.3.), which interact with dynamin at different domains and have a range of inhibitory potencies. Dynasore has been used widely and has allowed for a better understanding of the role of dynamin in various cellular processes. Dynasore validates the classical functions of dynamin, such as preventing receptor mediated internalisation [158] and even preventing HIV viral entry for some viral strains [147]. Dynasore treatment is highly disruptive to the formation of lamellipodia at the leading edge of migrating cells and therefore this inhibitor helps to understand the involvement of dynamin with cytoskeletal rearrangements [184]. For example dynasore increases the rate of neurotransmitter exocytosis in neuronal cells, but it also increases intracellular calcium concentrations. These increases intracellular calcium were only partially reversed by removal of calcium from the assay buffer suggesting dynasore may have some as yet unidentified effect on calcium mobilisation [201]. Dyngo-4a was discovered as a result of structure activity modification of dynasore and is a close structural analogue [202]. Dyngo-4a increases GTPase and RME inhibitory potency and acts as an inhibitor of Botulinum toxin, by inhibiting dynamin mediated endocytosis [142].

Table 1.3: Dynamin inhibitors and their properties. Dynamin inhibitor binding data is derived indirectly from observations of their inhibitory kinetics and their ability to prevent dynamin interaction with other cytoplasmic molecules. PH– Pleckstins homology, GA- GTPase allosteric, DA– Dynamin assembly (middle), nd – not determined.

Compound series	Compound	Binding locus	Determination of binding	Dynamin 2 GTPase IC ₅₀ (μM)	RME IC ₅₀ (μM)	Ring stabiliser?	References
Longchain ammonium salts	MiTMAb	PH	Compounds shown to be non-competitive GTPase inhibitors and capable of preventing PL binding to PH. No crystal data available	3.15 ± 0.64	20.9	No	[202-204]
	OctMAb	PH		1.9 ± 0.24	16	No	
	Mystric acid	PH		>300	>300	No	
Imino-chromines	Iminodyn 22	GA	Compounds shown to be uncompetitive with GTP and therefore to bind to 'GTPase allosteric domain' theorised to be at the GTPase domain due to uncompetitive nature.	0.39±0.15	10.7 ± 4.5	Yes	[202, 205]
	Iminodyn 17	GA		0.44±0.19	>1300	Yes	
Indoles	Dynole 34-2	GA	Compounds shown to be uncompetitive with GTP and therefore to bind to 'GTPase allosteric domain' theorised to be at the GTPase domain due to uncompetitive nature.	1.30 ± 0.30	5.0 ± 0.9	No	[202, 206]
	Dynole 31-2	nd		>> 300	Not determined	No	
N'-substituted 2-naphtho-hydrazides	Dynasore	GA	GTPase allosteric based on non-competitive inhibition. Does not inhibit lipid or GRB2 binding in vitro. Thought to occur at GTPase domain due to inhibitory effect on isolated Dyn 1 and 2 GTPase domains.	15	79.3 ± 1.3	Yes	[142, 158, 201, 202, 207, 208]
	Dyngo-4a	GA		0.3	16.0 ± 1.2	Yes	
Dimeric Tyrphostins	Bis-T	GA/DA	GTPase 'Allosteric' site – non-competitive binding. Acts upon Dynamin assembly domain.	1.7	~100	Yes	[209]

The McClusky group, who identified dyngo-4a, also described numerous other chemically diverse dynamin inhibitors, including dimeric tyrphostin 'bis-T' [209], longchain ammonium salts MiTMAB and OctMAB [203, 204], iminochromene [205] and indole species [206]. All these inhibitors were designed from libraries of modified small molecule precursors against dynamin GTPase function and as such can all affect internalisation. The way the molecules interact with dynamin results in some differences in function however; inhibitor-protein interactions are important in determining binding site interactions and mechanisms for allosteric interactions and are ideally determined by *in silico* or co-crystallisation and X-ray crystallography. In the case of the dynamin inhibitors, the binding to dynamin is not of sufficiently high affinity to allow co-crystallisation and no *in silico* models have been presented in the literature. The binding mechanisms for the inhibitors are putative and based on their ability to block phospholipid binding, as for MiTMAB and OctMAB or their kinetics during GTPase inhibition. Dynasore, dyngo-4a and bis-t are non-competitive inhibitors of GTPase function, which suggests binding to a site allosteric to the GTP turnover active site. Dynasore does not prevent lipid binding and has been shown to affect isolated GTPase domains, which places its binding zone in GTPase allosteric locus. Dynole 34-2 and iminodyn 22 are uncompetitive inhibitors [210], which means they bind the enzyme-substrate complex placing their binding site in close proximity to GTP at the GTPase binding site. Whilst this binding identification is crude, it offers information that might allow a better understanding of the function of dynamin in migration, particularly, as these inhibitors have a range of GTPase and RME potencies. Another point of comparison is the ability of these inhibitors to stabilise dynamin rings, which has recently been published in a patent application by Robinson *et al.* of McCluskeys group [202].

Dynasore has been shown to prevent CCR2 [178] and CCR5 [134] internalisation in THP-1 cells which demonstrates that this process occurs via dynamin interaction with the cell membrane in this cell line and suggests, that dynamin is present at the locus of CCR5 during receptor activation. There is little information regarding the role of dynamin in migration of highly mobile cells, such as monocytes and leukocytes, however. Considering the differential roles of PI3K and cell polarisation in these cells dynamin function with regard to chemotaxis makes an interesting

point of investigation. The range of dynamin inhibitors with their differential effects on dynamin may allow specific interactions within signal transduction to be deduced. For example MiTMAB and OctMAB, which bind dynamin at the pleckstin homology domain, would be expected to inhibit localisation with PIP₂, both compounds inhibit migration in fibroblasts but in THP-1 cells where PI3K mediated polarisation is not required for migration the effect might be different.

The final consideration regarding the dynamin inhibitors is that they were all screened for or rationally designed against dynamin GTPase function. Determining if dynamin GTPase potency correlates with any observed effect on migration will be an important step in determining the relationship between the protein and chemotaxis. If GTPase potency is shown not to correlate with any observed effects, then the other known traits of the inhibitors, such as protein binding location, receptor mediated internalisation potency or their ability to stabilise dynamin rings may offer insights in to dynamin function with regard to CCR5 signalling to migration.

1.13. Conclusions

The dysregulation of migration is causative to numerous disease states that make an understanding of the governing processes critical for the identification of potential therapeutical targets for these diseases. The chemokine receptor CCR5 is directly linked to inflammatory and metastatic diseases, but is also widely characterised intra- and extracellularly due to its involvement with HIV viral entry. This makes CCR5 a particularly good model system in which the specifics of cell migration can be determined with numerous cell lines available which either endogenously express CCR5 (THP-1 cells and activated PBLs) or are stably transfected with the receptor DNA . A large amount of research has been undertaken over the last decade to describe the cytoplasmic signalling events which govern cellular processes downstream of activated CCR5, there are still many contradictory or unresolved areas to be explored. There is evidence to support that the signalling from CCR5 to migration could be G α , G $\beta\gamma$ or arrestin dependent and that CCR5 binding proteins, such as JAK2, might play a role as well. Many of these contradicting results in the literature, such as the role of G $\beta\gamma$, non-receptor tyrosine

kinase signalling and PI3K mediated cell polarisation, may be cell type rather than receptor specific, but without further investigation this cannot be said for sure. The fact that PI3K is not required for THP-1 cell migration for example has not been directly attributed to CCR5 signalling nor has it been pharmacologically investigated to determine if small molecule inhibition can replicate the proteomic observations. Other protein targets known to be involved in migration, such as dynamin and Grb2, have not been well characterised for their interaction with CCR5. There are a large number of diverse inhibitors available for use against these proteins and small molecules still represent the most realistic route to intracellular signal transduction inhibition with potential therapeutic effects. Despite advances in antibody based pharmaceuticals and the potential for gene targeted therapeutics based around RNA interference, these methods are likely to be limited due to delivery problems. Exploration of CCR5 signalling to chemotaxis with cell permeable small molecule inhibitors has the advantage over proteomic techniques of identifying such mechanisms in that the 'targetability' of a process is validated as it is described. Knockdown or over expression of proteins can reveal potential drug targets but says nothing about how accessible the interaction is pharmacologically. For this reason the primary method of investigation of the signalling events will be through small molecular inhibition. These inhibitors will be used in an attempt to clarify cell type specific from receptor specific events and to examine the roles of poorly characterised proteins such as Grb2 in an attempt to identify novel, small molecule targetable processes which can down regulate migration. This thesis will outline protein interactions of interest that can then become the focus of targeted proteomic analysis.

1.14. Research objectives

Pharmacological disruption of chemokine receptor mediated processes represents a path to therapeutic intervention in many diseases. Despite this there are no chemokine receptor based therapies for immunological or metastatic disease available, the reason for this is, in part, due to the lack of understanding of chemokine receptor mediated signalling. It has been shown that signalling from CCR5 to chemotaxis is far from resolved due in part to the large number of

protein interactions which can potentially be involved. The introduction demonstrated that based on the current understanding CCR5 signal transduction could be attributed wholly to $G\alpha$, $G\beta\gamma$ and arrestin dependent signal transduction. Other proteins such as FROUNT, SFKs and PI3K may also be linked to CCR5 mediated migration perhaps through interactions with poorly characterised CCR5 interacting proteins such as JAK2. Other proteins prominently involved in migration downstream of other receptor types such as dynamin and Grb2 do not have a clearly defined relationship with CCR5 or chemokine receptors generally. Clearly all of these proteins cannot simultaneously be involved; identifying which of these interactions are receptor, receptor family or cell type specific will form the focus of this thesis. Resolving the specifics of chemokine receptor signalling by small molecule disruption of protein signalling and comparative analysis with other chemokine receptor signalling systems has the potential to identify novel receptor specific targets for disruption of chemokine receptor function. In order to achieve this, the following experimentation will be undertaken:

CHAPTER 3: An investigation into the chemotactic responses stimulated by various CCR5 ligands in the THP-1 cell line and whether CCR5 specific responses can be resolved through the use of small molecule chemokine receptor antagonists.

Chapter 4: Will explore the identification of 'way points' in CCR5 mediated THP-1 cell migration so that the conflicting reports in the scientific literature can be clarified and used to direct further research.

Chapter 5: An investigation into the potential role of JAK/STAT signalling as a player in cytoplasmic signal transduction to migration outside of their role as transcription factors.

Chapter 6: In chapter six proteins which have been shown to be important in migration in other receptor signalling systems such as PI3K, dynamin and Grb2 will be investigated in relation to CCR5 mediated migration.

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 are derived from a one year old male donor with acute leukaemia. THP-1 cells are known to represent a good model for healthy monocyte behaviour and 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 (PAA, Yeovil, UK) supplemented with 10% v/v Foetal calf serum (FCS) (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), 100 µM non-essential amino acids (Gibco) and 2 mM glutamine (Invitrogen).

2.1.2. HeLa.RC49 cervical cancer fibroblasts

HeLa.RC49 cells represent immortalised fibroblasts which stably express human CCR5 with negligible expression levels of non-transfected chemokine receptors and were acquired from D. Kabat [211]. HeLa.RC49 are cells that contain human signalling machinery in conjunction with high levels of human CCR5 receptors and have proven robust in *in vitro* assays. Briefly, HI-R clone of HeLa-CD4 cells identified with low expression of CD4 were transfected with the retroviral vector SFF-CCR5. CCR5 expression was validated by susceptibility to infection by the Ba-L M-tropic HIV-1 isolate. The RC49 clone was shown to express the highest level of CCR5 membrane receptors. HeLa.RC49 cells were grown in complete Dulbecco's Modified Eagle's Medium (DMEM) (PAA) (10% v/v FCS, 100 U/mL penicillin and 100 µg/mL streptomycin, 100 µM non-essential amino acids and 2 mM glutamine).

2.1.3. CHO.CCR5 stably transfected Chinese Hamster Ovary fibroblasts

Chinese hamster ovary (CHO) cells have proven to be easy to culture, fast growing and, due to their relatively large size, excellent for microscopy. CHO.CCR5 cells were acquired from J. McKeating, Reading. CHO were transfected with pcDNA3 encoding Human CCR5 and selected for stable expression in 10% FCS DMEM with glutamine (2 mM) in the presence of the selective aminoglycoside antibiotic G418 (Invitrogen) (400 µg/mL). CHO.CCR5 cells were cultured in complete DMEM (10% v/v FCS, 100 U/mL penicillin and 100 µg/mL streptomycin, 100 µM non-essential amino acids and 2 mM glutamine supplemented with 400 µg/mL G418 to ensure continued expression of CCR5.

2.1.4. Routine tissue culture procedures for cell lines

Both adherent and suspension cell lines were cultured in 75cm² flasks (Corning/ SPL Life Sciences) in a humidified atmosphere at 37°C with 5% CO₂. 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 15-20 minutes at 37°C and 5% CO₂. After this period, cells were suspended by gentle agitation of the flask. Cells were then centrifuged at 700 g for 5 minutes and resuspended in the 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 1 week or until cell density reached 6x10⁴/mL (whichever was sooner) at which point cell density was reduced to 2x10⁴/mL by removal of suspended cells and addition of fresh media. Where cells were to be cryopreserved, 1x10⁶ cells were centrifuged, resuspended into 1 ml of 10% (v/v) dimethyl sulfoxide (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 24hr before long term storage in liquid nitrogen at -196 °C.

2.1.5. Peripheral Blood Mononuclear Cell (PBMC) collection

Venous blood from healthy human volunteers was collected into falcon tubes containing approximately 10% sodium citrate anti-coagulant (4% w/v) then mixed with an equal volume of HEPES buffered saline solution (HBSS). Peripheral blood mononuclear Cells (PBMCs) were isolated by centrifugation through Ficoll Paque Plus (General) (15mL/25ml of blood) at 400g for 35 min RT. The PBMC containing fraction was washed twice in pre-warmed HBSS before re-suspension in simple RPMI-1640.

2.1.5.1. Isolation and Activation of Peripheral Blood Lymphocytes

Cells were added to a 75cm² flask for adherence depletion of monocytes. After 4hr non-adherent cells were removed, washed in simple RPMI-1640, then resuspended at $1 \times 10^6 \text{ mL}^{-1}$ in complete RPMI-1640 (10% FCS, 1% NEAA, 2 mM glutamine). Peripheral blood lymphocytes (PBLs) were activated by addition of (30 mg/mL) concanavalin A (Con A) (Sigma-Aldrich) and 200 $\mu\text{g/mL}$ IL-2 (Peprotech) and incubated for at least 10 days before use in chemotaxis and calcium flux assays. PBLs were cultured by addition of 1 mL of complete RPMI-1640 at 2 day intervals after activation was complete. Due to variability between donor PBLs all experiments using these cells represent at least 3 donors. Individual donor data (n=1) represents the mean values obtained from at least 3 independent experiments.

2.2. Small molecule and oligomeric inhibitors

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

Table 2.1. JAK/STAT inhibitors:

Inhibitor	Supplier	Stock Conc/Vehicle	Working Conc
Cucurbitacin I	Tocris Bioscience	20 mM ethanol	10 μ M
JAK2 inhibitor II	Calbiochem	100 mM DMSO	50 μ M
STAT3 inhibitor III	Calbiochem	100 mM DMSO	10 μ M
STAT3 inhibitor VIII	Calbiochem	100 mM DMSO	10 μ M

Table 2.2. PI3K inhibitors:

Inhibitor	Supplier	Stock Conc/Vehicle	Working Conc
LY294002	Abcam	25 mM DMSO	10-625 μ M
Wortmannin	SigmaAldrich	50 μ M DMSO	2-100 nM
AS605240	Selleck Biochem	1 mM DMSO	5-1000 nM

Table 2.3. Cytoskeletal inhibitors:

Inhibitor	Supplier	Stock Conc/Vehicle	Working Conc
Nocodazole	Tocris Bioscience	25 mM DMSO	10-625 μ M
Cytochalasin D	Tocris Bioscience	300 μ M DMSO	3-7.5 μ M

Table 2.4. Kinase inhibitors:

Inhibitor	Supplier	Stock Conc/Vehicle	Working Conc
PD98059	Calbiochem	25 mM DMSO	2.5 μ M
SB203580	Calbiochem	2 mM DMSO	2 μ M
β ARK1 inhibitor	Calbiochem	19 mM DMSO	190 μ M
Bisindolylmaleimide I hydrochloride	Calbiochem	2.5 mM DMSO	2.5 μ M
PKC ζ pseudo substrate inhibitor	Calbiochem	1 mM DMSO	10 μ M
Bosutinib	Selleck Biochem	10 mM DMSO	10 μ M
Farnesylthiosalicylic acid	Abcam	12.5 mM DMSO	12.5-250 μ M
CGP078850	Novartis	50 mM DMSO	50-500 μ M

Table 2.5. Endocytosis inhibitors:

Inhibitor	Supplier	Stock Conc/Vehicle	Working Conc
Sucrose	SigmaAldrich	4M water	0.4 M
Filipin	Tocris	15 mM DMSO	15 μ M
Pit stop 2	Abcam	30 mM DMSO	30 μ M
Pit stop 2 negative control	Abcam	30 mM DMSO	30 μ M
MiTMAB	Ascent Scientific	10 mM DMSO	10 μ M
OcTMAB	Ascent Scientific	5 mM DMSO	5 μ M
Promystric acid	Ascent Scientific	15 mM DMSO	15 μ M
Iminodyn 22	Ascent Scientific	1 mM DMSO	1 μ M
Iminodyn 17	Ascent Scientific	1 mM DMSO	1 μ M
Dynole 34-2	Ascent Scientific	15 mM DMSO	15 μ M
Dynole 31-2	Ascent Scientific	15 mM DMSO	15 μ M
Dynasore	Abcam	80 mM DMSO	15-80 μ M
Dyngo-4a	Abcam	10 mM	5-80 μ M

Table 2.6. Chemokine receptor antagonists:

Inhibitor	Supplier	Stock Conc/Vehicle	Working Conc
Maraviroc	Tocris Bioscience	500 μ M DMSO	500 nM
J113863	Tocris Bioscience	10 mM ethanol	1-10000 nM

2.2.1. Chemokine Receptor Ligands

The chemokine used for CCR5/CCR1 activation was CCL3 (D26A), which was generously donated by Lloyd Czaplewski of British Biotech. The isoform has been referred to in a previous publication as CCL3 (2–70) (D26A) in comparison with the full gene sequence for CCL3. CCL3 (2–70) (D26A) is referred to in this text as CCL3. CCR5 agonist CCL4 (MIP1 β) (Peprotech, New Jersey, USA) stock was made up to 10 μ M for use in chemotaxis assays at concentration of 1nM. CXCR3 ligand CXCL11 (ITAC) (Peprotech, New Jersey, USA) stock was made up to 1 μ M in purified water for use in chemo attractant assays at 20nM. CXCR4 ligand CXCL12 (SDF1) (Peprotech, New Jersey, USA) stock was made up to 10 μ g per mL in purified water for use at working concentration of 1 μ g per mL in chemo attractant assays (about 10nM). Chemokine ligands CCL2, CCL5 and CCL8 (all Peprotech) stock solutions were made up to 1 mM in purified water for use at a working concentration of 1 nM.

2.3. Antibodies

Table 2.7: Primary and secondary antibodies used for experiments. IF= Immunofluorescence, WB= Western Blotting.

Primary Antibody	Supplier	Target	Dilution factor/ Assay
HEK/1/85a/7a rat monoclonal	A gift from J.A.McKeating, Reading	Human CCR5	1:100
ABT49 rabbit poly-clonal	Millipore	Human Dynamin 2	1:500 IF 1:1000 WB
SC-8034 mouse monoclonal	SantaCruz	AA 54-164 Grb2 central domain.	1:500 IF
ab49876 mouse monoclonal	Abcam	AA 200-217 Grb2 C-terminus	1:500 IF 1:1000 WB
Secondary	Supplier	Assay use	Dilution factor
Goat anti-mouse FITC	Sigma-Aldrich	IF	1:1000
Goat anti-rabbit Alexa-514	Invitrogen	IF	1:1000
Goat anti-rabbit Alexa-595	Invitrogen	IF	1:1000
Anti rat IgG horseradise peroxidase	Sigma-Aldrich	WB	1:10000
Anti rabbit IgG horeradish peroxidase	Sigma-Aldrich	WB	1:10000
Anti-mouse IgG Horseradish peroxidase	Sigma-Aldrich	WB	1:10000

2.4. Plasmid DNA

The pEGFP arrestin 2/3 plasmids were produced by cloning relevant arrestin DNA into Hind III/Apa I digested pEGFP-N1 plasmids (Clontech, Saint-Germain-en-Laye, France) as described and validated [212, 213] and were a gift from E. Kelly (Bristol). pEGFP.C2 plasmids (Clontech) were used as mock transfection controls. Dynamin K44A plasmids were a generous gift from S. Mundell (Bristol).

2.4.1 Preparation of plasmid DNA from bacterial colonies

E. coli DH5 α F¹ (Invitrogen) were transformed with the required plasmid DNA to allow amplification of plasmids. 50 μ L of *E. coli DH5 α F¹* were thawed in iced-water and gently broken down with a pipette to form homogenous slurry. 1-5 μ g 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 sec before 2 min incubation in iced-water. 1 mL of pre-warmed Lennox Broth (LB) was added to the heat shocked *E. coli* before incubation in a rotary shaker at 37°C, 200 rpm for 2 hours. 25 μ l of the resulting bacterial broth was spread on prepared LB agar plates containing kanamycin (100 μ g/ ml) and grown, upside-down, overnight at 37°C. Viable colonies were picked then incubated overnight in 20 ml LB containing kanamycin (100 μ g/ ml). The cultured bacterial broth was then frozen down at -80°C or used for plasmid amplification described below.

2.4.2. Amplification of Plasmid DNA

Plasmid DNA was purified using the Qiagen MIDI kit (Qiagen). The relevant transformed DH5 α F¹ strain was grown overnight and the bacterial broth was centrifuged at 4000rpm for 10 minutes. The supernatant was removed and 4mL of P1 buffer containing RNAase was added to resuspend the pellet. 4mL 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. 4mL of P3 buffer was added to precipitate protein then the solution was centrifuges at 4000rpm for 10 minutes.

Supernatant was filtered through an activated Qiagen column, filtered and then flushed with 10mL QC buffer. Plasmid DNA was then eluted with 5mL QF buffer. 3.5 mL propan-2-ol was added to the eluent to precipitate DNA during centrifugation for 1h at 4000rpm. The supernatant was removed and the pellet washed in ethanol before being dried and dissolved in 1mL deionised water. Plasmid DNA purity was determined by gel electrophoresis after digestion with EcoRI restriction enzyme (Promega). Plasmid DNA concentration was determined by absorbance at 260 nm using the Nanodrop spectrometer system (Thermo Scientific).

2.5. Electroporation transfection

Cells were counted and spun down to give 1×10^6 cells per tube. Cells were resuspended in 105 μ L simple RPMI or 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 μ L t-RNA and 1 μ g plasmid DNA and 1% DMSO was added before incubation at room temperature for 20-30minutes. Cells were resuspended and 100 μ L of the transfection mixture was added to a 0.2cm transfection cuvette and electroporated at the relevant setting (U-001 for THP-1). 100 μ L of simple RPMI was added to the cuvette and the contents incubated at room temperature for 10 minutes. The solution was removed with a pipette and transferred to a pre-incubated 6-well plate containing the relevant growth media. Cells were incubated for 48h before assessment of transfection efficiency by fluorescence microscopy. For 0.4cm cuvettes all volumes are increased by factor 2.5.

2.6. Calcium flux

Adherent cell lines were harvested by addition of PBS containing 25 mM EDTA. THP-1 cells were collected, centrifuged at 2000 g for 5 minutes. All cells were resuspended in calcium flux buffer (137 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 1.5 mM CaCl_2 , 10mM HEPES pH 7.4, 25mM D-Glucose. Cells were spun down and washed twice more in calcium flux buffer before resuspension in 1mL of calcium flux buffer to give a concentration of 1×10^6 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). 2 μ M of Fura-2 was added to all cell samples before incubation at 37°C, CO₂ 5% for 30 minutes. Cells were then centrifuged at 2000 g and washed with 1mL calcium flux buffer 3 times. 100 μ L of cell was added to each well of a black, opaque, reader plate (Fischer Scientific, UK) before calcium flux assessment by addition of different agonist/antagonist injected directly into the wells using the 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 the ratio of the fluorescence detected at 340 nm/380 nm. The change in fluorescence was calculated as the difference between the pre-stimulated 340/380 nm ratio and the peak value after stimulation.

2.7. Migration assays

2.7.1. Transwell chemotaxis assay

Chemotaxis assays for THP-1 and PBLs were carried out in ChemoTX 5 μ m pore transwell chemotaxis plates (Neuroprobe Inc, USA). Wells to be used in the chemotaxis plate were blocked with 30 μ L blocking buffer (1% BSA in simple RPMI) for at least 30 minutes at room temperature. Chemokine solutions were prepared at 1-5 nM, depending on assay requirements, in working buffer (0.1% BSA in simple RPMI). THP-1 cells were micro-centrifuged at 5000 rpm for 5 mins, washed once in simple RPMI and resuspended in 39 μ L working buffer to give 25×10^4 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 30mins at 37°C, 5% CO₂ for 0.5hr. The blocking buffer was removed from the plate and replaced with 31 μ L of the relevant chemokine solution (working buffer for unstimulated samples). The membrane was attached and 20 μ L of cell suspension was added to the top surface. The plate was

incubated in a fully humidified chamber for 5h before membrane was dried and removed and the cells in each well counted with a haemocytometer.

2.7.2. Scratch assay

Initially sterile 6 well plates were prepared by marking the outside of the bottom surface with two parallel lines in indelible marker per well which forms the reference points for wound healing quantification- this must be done before addition of cells.

Adherent cells were cultured until around 90% confluence was reached and resuspended by incubation with PBS with 0.25 mM EDTA for 15 mins. Cells were washed in PBS then resuspended in 12 mL of complete DMEM at a density of 5×10^5 cells per mL. Cells were pipetted into pre-marked 6 well plates (2 mL per well) and incubated for 24 hours at 37°C to allow adherence and spreading of the cells. Scratches were made in the cell layer perpendicular to the reference lines using a 200µL pipette tip drawn along the side of the plate lid. The complete medium was then removed and the cells washed twice in PBS to remove loosened and suspended cells. 1 mL of simple DMEM containing 1% FCS was then placed in each well and the wounds were imaged in duplicate using a Leica inverted microscope in bright field mode in such a way that the horizontal reference line was visible in all images.

The relevant chemokine/inhibitor mix was prepared in another mL of 1% FCS DMEM which was added to the wells giving a total volume of 2 mL. Images were then collected at 6, 24 and 48h after addition of treatments. Wound healing was quantified using Photoshop CS5 (Adobe) by aligning the horizontal reference line to vertical 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 pixel width between the two edges of the scratch could then be determined. For time point images the transformation setting used for the t=0 images were used and perpendicular pixel width re-determined as described.

2.8. Imaging techniques

2.8.1. Phalloidin actin stain

CHO.CCR5 or HeLa.RC49 cells were split at around 95% confluence with 4ml PBS EDTA and 0.5mL was placed over washed coverslips in a 6 well plate. Cells were incubated for 24 hours to allow the cells to become adherent. Once 95% confluence was reached, the inhibitors were added at working concentrations and left for 30minutes. CCL3 was then added to relevant wells to a concentration of 100 nM and cells were then incubated for 1-2hr. Cells were washed twice in PBS and fixed by adding 500 μ L ice cold 4% formaldehyde solution for 10 mins. Cells were washed twice in PBS then washed 3 times for 5 minutes in 0.1% Triton X-100 (FischerBioTech) solution. Cells were washed twice in PBS and then 2 μ L of Alexa Fluor 488 tagged phalloidin (Invitrogen) was added to 200 μ L PBS and placed onto the slides before incubation in the dark for 20 minutes. Cells were washed twice in PBS and mounted onto glass slides with Citifluor AF1 (Citifluor, UK), a fluorescence stabilising mounting medium. Cells were analysed immediately by fluorescence microscopy.

2.8.2. Antibody staining Immunofluorescence

Cells were seeded on coverslips overnight as described above. Once 95% confluence was reached the inhibitors were added at working concentrations and left for 30minutes. Where necessary CCL3 was added at 100 nM concentration and left for 1-2hrs. Growth medium was removed and cells were washed once in PBS before being fixed with 500 μ L ice cold 4% formaldehyde solution. Fixing solution was removed and cells were permeabilised by 3 times addition of 0.1% Triton X-100 (FischerBioTech) solution. The cells are washed with 1x PBS and incubated overnight at 4 °C with the primary antibodies in 1x PBS supplemented with 4 % BSA (Sigma). Where dual antibody stains were required both primary antibodies were added to the solution. The following day the cells are washed 4 times for 10 minutes each with 1x PBS and incubated for 1.5 hours at room temperature with the secondary antibodies in 1x PBS

supplemented with 4% BSA. Where dual stains were required both secondary antibodies were added at the same time. Cells were washed twice in 1xPBS. If an actin stain was required Alexa Fluor 488 tagged phalloidin was added at this point as described above. Post actin staining, and if actin staining was not required a final wash of 1xPBS containing 0.6 μ M DAPI nuclear stain was added to the cells before mounting. Cells were mounted onto glass slides with Citiflour AF1 (Citiflour, UK). Cells were analysed immediately by fluorescence microscopy.

For THP-1 cell staining the procedure above was followed although cells were prepared in suspension in eppendorfs tubes and centrifuged between treatments and washes. Mounting of THP-1 cells was achieved by resuspending pelleted cells in 100 μ L after the final wash in PBS then pipetting 20 μ L of the resulting suspension into a glass cover slip. These coverslips were allowed to dry in a laminar airflow hood then mounted directly onto a glass slide using Citiflour AF1 mounting medium.

2.8.3. CCR5 receptor Internalisation

Cells were seeded overnight onto coverslips. Once 95% confluence was reached the inhibitors were added at working concentrations and left for 30minutes. Cells were then stimulated with 100 nM CCL3 and incubated at 37°C, 5% CO₂ for 1hr. Cells were washed once with PBS and HEK/1/85a/7a (1:100) in 1 mL simple DMEM was placed onto the cells which were then incubated at 4°C for 2hrs. Cells were washed 4 times for 10 minutes in PBS before fluorescently tagged secondary Ab (1:1000) was added in 1 mL PBS and incubated for 1hr at 4°C. Cells were washed three times in PBS and mounted onto glass slides with Citiflour AF1 (Citiflour, UK). Cells were analysed immediately by fluorescence microscopy.

2.8.4. Microscopy

Fluorescently stained slides were imaged using either a Leica wide field fluorescence microscope fitted with 10, 40 and 63x inverted air objectives fitted with a colour CCD camera, or, a Zeiss upright wide field fluorescence microscope fitted with 10, 40, 63 and 100x oil objectives fitted with a monochrome CCD. Leica images were captured and analysed using the Leica imaging suite software (overlays represent true colours) and Zeiss images were captured and analysed using the Axio2 imaging software (all colours assigned post capture). Confocal images were obtained using a Leica TCS SP2 UV system laser scanning microscope. Images represent a single z axis slice of around 5 microns. All immunofluorescence was validated by testing for non-specific secondary anti-body binding.

2.9. Flow cytometry

THP-1 cells were washed in PBS and resuspended to give 1 mL with 1×10^6 /mL. Cells were treated with 2 μ M cytochalasin D for 0.5hr or left untreated as controls before activation with 100 nM CCL3. Cells were then permeabilised, as described above, then washed and resuspended in 500 μ L PBS and treated with 10 μ L Alexafluor-488 tagged phalloidin for 20 mins. Cells were washed twice and resuspended in 500 μ L PBS after which a 10 μ L aliquot of the samples was viewed by fluorescence microscopy to ensure staining could be observed. Differences in cellular fluorescence were then determined using an Accuri C6 flow cytometer (BDBiosciences) measuring 10,000 cellular events per sample. Data was analysed using C-Flow software (BDBiosciences).

2.10. Westernblotting

2.10.1. Sample preparation

5×10^5 cells were spun down at 2000 g for 5 mins 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 μ L of lysis/sample buffer (4% SDS, 0.02% bromophenol blue, 20% glycerol, 1.5 mM DTT, 80 mM Tris, pH 6.8) was added and mixed until a homogenous consistency was reached. Cell lysing was facilitated by sonication (3 pulses of 3 seconds at 90% amplitude and 0.9 pulse). The sonicated suspension was then placed in boiling water for 5 minutes to denature proteins further. The lysis mixture was then immediately spun down at 14000 g for ten minutes and the supernatant was removed in preparation for gel electrophoresis, the pellet was discarded.

2.10.2. 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% of the 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 μ L of sample was loaded into the stacking phase wells with a Hamilton microsyringe along with 5 μ L broad-range protein ladder (BIO-RAD, UK). Unused wells were loaded with 20 μ L 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 was increased to 50 mA constant until the elution front ran off the gel at which point electrophoresis was complete.

2.10.3. 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 deionised water. 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 (BIO-RAD semi-dry transfer kit). The transfer was undertaken at 15 volts constant for 45minutes. Successful protein transfer was evaluated by the transfer of the protein marker to the nitrocellulose membrane.

2.10.4. 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 1hr at room temperature. Blocking buffer was then removed and the relevant primary antibody (see table 2.7.) was diluted in blocking buffer before overnight rolled-agitation. The following day the primary anti-body was removed and the membrane washed 3 times for 10 min with PBS-T before addition of the relevant secondary peroxidase tagged Ab was added at 1:10000 in 10 mL blocking buffer. After 1hr roller-agitation the secondary antibody solution was removed and the membrane was washed 3 times for 10 minutes in a tray with gentle shaking to facilitate flattening. Proteins were visualised by addition of 2-sprays of the one-pot RapisStep ECL (Calbiochem) 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).

2.11. Enzyme linked immunosorbance assay

The InstantOne™ STAT3 phosphotyrosine 705 ELISA (eBiosciences, UK) was used to determine the effectiveness of JAK and STAT inhibitors. The ELISA kit contained all the required sample and lysis buffers and antibodies. HeLa.RC49 cells were grown to 95% confluence and harvested using PBS containing 25 mM EDTA to give 1×10^6 cells. Cells were centrifuged at 700 g for 5 minutes and resuspended in simple DMEM before treatment with 10 μ M Stat inhibitors to final volume of 200 μ L. Controls cells were treated with the relevant concentration of DMSO vehicle and all treatments were then incubated at 37°C, 5% CO₂ for 30 minutes. After incubation 0.5 μ L of IL-6 (40 ng/mL) (Peprotech) was added to relevant cells and incubated for 15 min. Cells were washed once in PBS and centrifuged at 700 g for 5 minutes and the supernatant was removed to leave a pellet. 50 μ L of the supplied 1x lysis buffer was added to the pellet and aspirated to mix before 10 minutes shaking at room temperature. 50 μ L of the lysate was added to the ELISA plate (except in –ve control wells) and 50 μ L of the supplied antibody cocktail was added to the lysate samples before shaking for 1hr at 140 rpm. Samples were then washed 3 times with the supplied wash buffer and 100 μ L of pre-equilibrated detection reagent was added to all wells and incubated at room temperature for 30 minutes. 100 μ L of the supplied stop reagent was added to all well and the plate was read at 450 nm using a BMG Labtech micro plate reader.

2.12. MTS cytotoxicity assay

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used to determine if compounds were cytotoxic. The assay is colourimetric and works on the principle that healthy cells can metabolise [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into its formazan product, which shows significantly higher absorbance at 490 nm than the parent. 2.5×10^5 cells were used per well for the MTS assay; cells were washed in PBS then made up the correct density so that 100 μ L could be placed in a 96-well plate. The relevant test compounds were added to the cells in duplicate the samples were incubated at 37°C, 5% CO₂ for 30 minutes. 10 μ L of the CellTiter 96 MTS solution

was added to the relevant wells (except background controls) and the plate was incubated for 5hr at after which the plate was read at 490 nm using a BMG Labtech Fluorostar microplate reading fluorometer.

2.13. Curve prediction and statistical analysis

All data were analysed using Graphpad Prism 5 and represent at least 3 independent experiments (unless stated otherwise). For human PBL experiments individual donor cells were used to perform 3 independent experiments and the mean result was taken as n=1, at least 3 donors were used. All concentration response sigmoidal curves used to calculate EC₅₀ and IC₅₀ values were fitted assuming a Hill coefficient of 1. Statistical significance were determined using unpaired students t-tests where applicable or one-way ANOVA analysis of variance followed by Bonferroni multiple comparison post-test where 3 or more data sets were to be analysed. A significance value of 95% was used with p values indicated as follows p≤0.05 = *, p≤0.01 = **, p≤0.001 =***. P values above 0.05 where therefore considered not significant (ns). All error bars represent standard error of the mean value (S.E.M)

CHAPTER 3: Determination of chemokine receptor mediated cellular responses in THP-1 cells

3.1. Introduction

The immortalised monocytic leukaemia cell line THP-1 has been used widely as a model for monocyte behaviour in *in vitro* assays and, despite some minor functional differences with primary monocytes, has been validated as a model for several human diseases [214, 215]. Proteomics analyses of THP-1 cells have revealed expression of numerous chemokine receptor families and isotypes including CCR5 [134, 178, 216-219] (Table 3.1).

Table 3.1: Chemokine receptors expressed in THP-1 cells

Receptor	Determined by	Reference
CCR1	Western blotting	[217, 218]
CCR2	Western Blotting	[178, 216]
CCR5	Western blotting	[134, 219]
CXCR4	Western blotting	[134, 220]

Due to the validity of the cell line as a model for disease, the fact that it is widely validated in *in vitro* chemotaxis assay and the fact that they express high levels of CCR5, THP-1 cells were used as the primary model system to investigate CCR5 mediated signalling events associated with migration stimulated by CCL3, 4 and 5. Specific chemokine receptor signalling stimulated by chemokines can be difficult to isolate however, due to the propensity of chemokines to either share more than one receptor [28, 221] or for activated receptors to transactivate dimeric or arrayed partners [84]. For this reason, proteomic receptor identification alone cannot predict THP-1 cellular responses to chemokines let alone attribute these responses to specific

receptors. Therefore it is important to identify THP-1 cellular response to CCL3/4/5 and then determine whether the responses specific to CCR5 can be determined experimentally, so that clear conclusions about the role of the individual receptors in chemotaxis can be reached.

The production of high affinity chemokine receptor antagonists, such as the CCR5 inhibitor maraviroc and the CCR1 antagonist J113863 allows the inhibition of specific receptors during stimulation with chemokines. The fact that chemokines are usually specific to a small number of well-characterised intra-motif chemokine receptor types should allow a functional profile of cellular responses to specific chemokines to be determined and compared to receptor types present on the cell surface. If, for example, the use of CCR1 antagonists allows signalling through CCR5 receptors to occur normally, then these antagonists can be used in all future experimentation to ensure that only CCR5 specific responses are observed.

3.2. Chapter Aims

In order to determine which characteristics of THP-1 cellular responses can be attributed to CCR5 signalling, analysis of responses in calcium flux and chemotaxis assays to a range of different chemokines can be determined. When these assays are performed in the presence of CCR1 and CCR5 receptor antagonists, then any observed alterations in cellular responses can be noted. The results of these experiments will allow conclusions to be drawn as to whether further experimental data can be related to CCR5 specific events. The experimentation in the presence of other chemokine receptor antagonists will be used to determine if CCR5 specific signalling can be isolated. If this is the case this will simplify the analysis of receptor mediated cellular responses observed in the rest of the thesis.

3.3. Results

3.3.1: The determination of basal responses to CCL3 stimulation

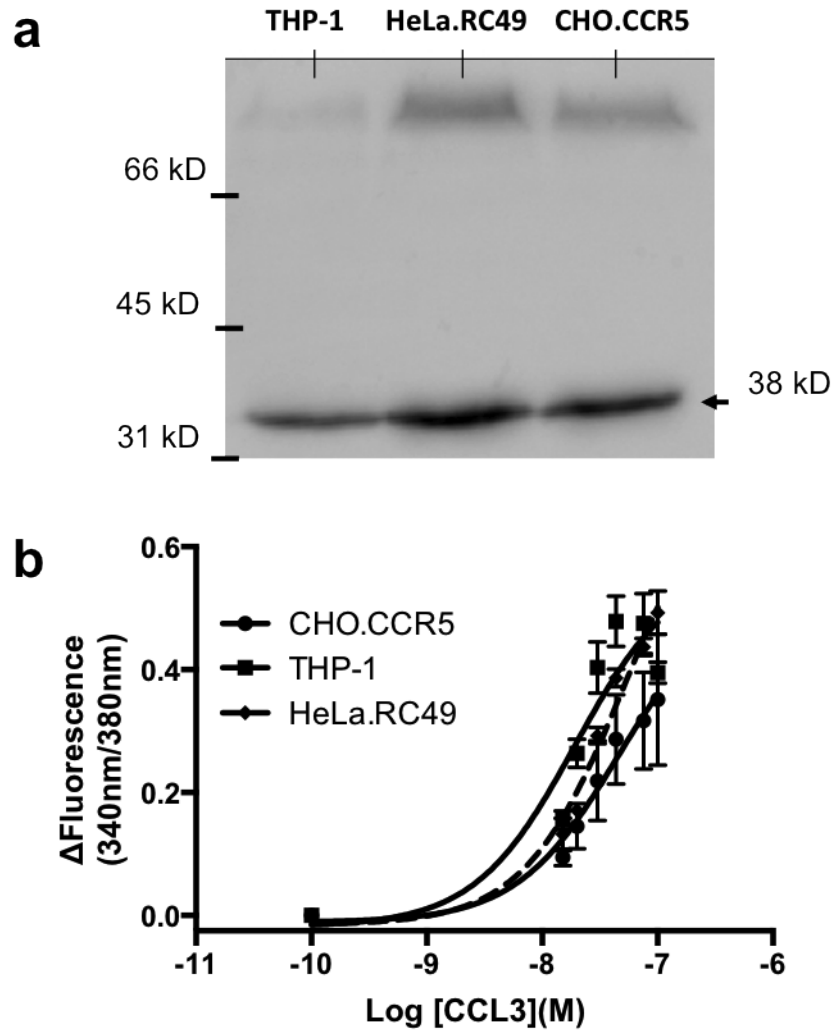


Figure 3.1: THP-1 cells express CCR5 and undergo calcium release upon CCL3 stimulation. (a) Western blotting reveals THP-1, HeLa.RC49 and CHO.CCR5 cells express the 38 kD GPCR CCR5 (characteristic double band shown). (b) Concentration response curve for increasing concentrations of CCL3 stimulating intracellular calcium release in THP-1, HeLa.RC49 and CHO.CCR5 cells. Results represent the mean \pm SEM of at least 3 independent experiments.

The model cell lines used in this thesis express CCR5 either endogenously or are stably transfected to express the CC-motif chemokine receptor CCR5 as determined by western blotting (Figure 3.1a). The 38 kD GPCR was identified with HEK/1/85a/7a rat anti-CCR5 Ab and showed two bands which are generally associated with a monomeric and a dimerised form of the receptor [6]. The β -chemokine receptor ligand CCL3, which is known as a CCR5 agonist but has also been shown to activate CCR1 [28], induces calcium release in THP-1, HeLa.RC49 and CHO.CCR5 cells in a concentration dependent fashion (Figure 3.1b). The EC_{50} for calcium release in this system was determined by sigmoidal concentration-response regression (assuming a Hill co-efficient of 1, see methods and materials) and calculated as $EC_{50} = 40.0 \pm 6.2$ nM. Since CCL3 activates both CCR5 and CCR1, and both receptors are present on THP-1 cells, the calculated EC_{50} must be taken as representative of the combined action of these receptors.

The Chemo-TX transwell chemotaxis assay was used to determine the migration response induced by CCL3 stimulation. THP-1 cells migrated towards a stimulus of CCL3 with maximum migration occurring at 1 nM CCL3 (Figure 3.2a). The migration response seen is typical of migrating cells activated via chemokines, with chemotaxis being concentration dependent up to an optimal level then dropping of steeply with further increases in stimulus [13, 39]. It was also noted that non-stimulated, or basal, migration always occurred, and was consistent at around $30 \times 10^4 \text{ mL}^{-1}$ cells in all assays performed. Basal migration is important to consider in migration assays as it can provide information about non-specific effects of treatments. DMSO is the primary vehicle for most inhibitors used in the thesis, so the effects of DMSO concentration on stimulated THP-1 cell migration were determined. DMSO has been shown to affect viability and protein expression levels at concentrations above 1% v/v (personal communication from Dr O'Connells research group) when incubated with cells for longer periods of time, but in the 5.5hr exposure of the migration assay concentrations up to 2.5% v/v had no significant effect.

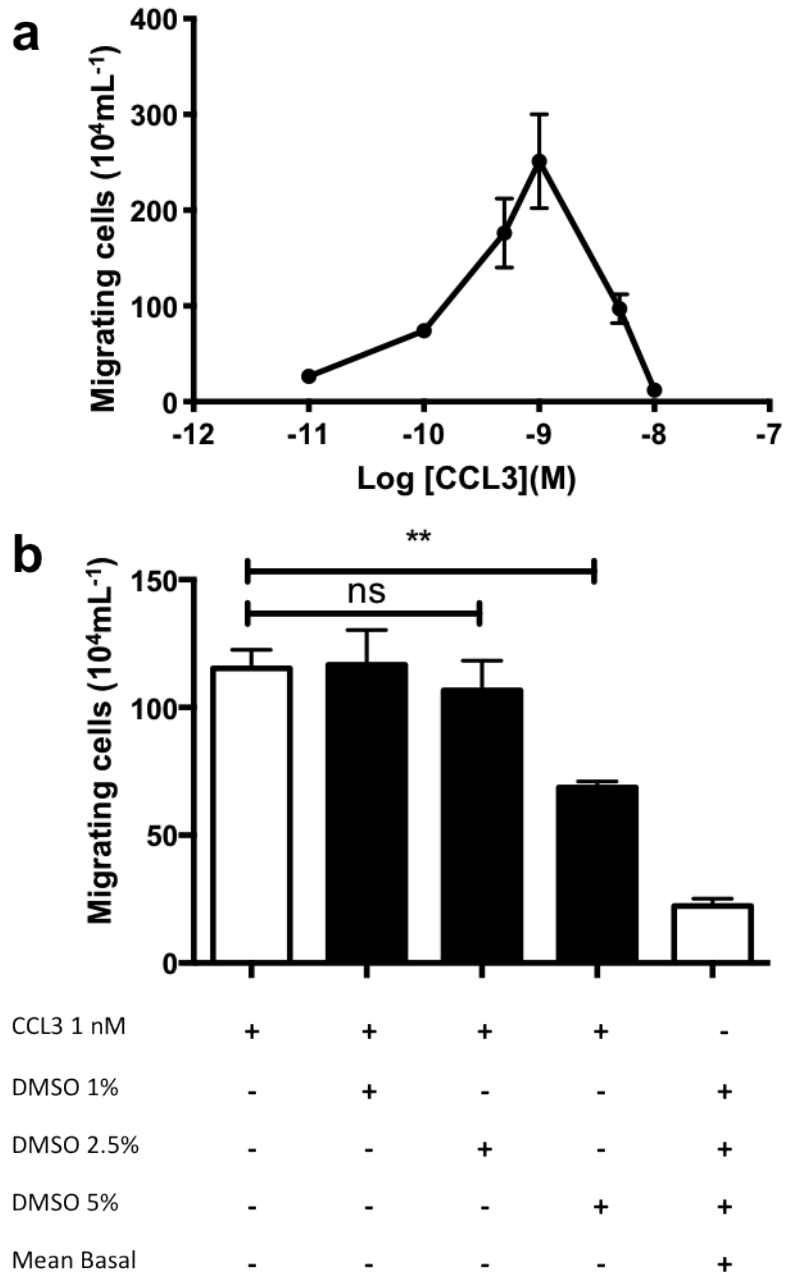


Figure 3.2: THP-1 cells migrate towards CCL3 in the Neuroprobe Chemo-TX transwell chemotaxis assay. (a) Migration of THP-1 cells towards CCL3 is concentration dependent up to a peak chemotaxis at 1 nM. (b) Concentrations of inhibitor vehicle DMSO significantly affect the ability of THP-1 to migrate towards 1 nM CCL3 at levels at and above 2.5% v/v concentration ($p \leq 0.01$ $n \geq 3$). Results represent the mean \pm SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison. ** = $p \leq 0.01$, ns = not significant.)

Increases in protein expression are unlikely to affect THP-1 migration, with the duration of the assay being shorter than the time frame for protein synthesis. At 5% v/v DMSO begins to affect migration in THP-1 cells and significantly reduces the ability of the cells to migrate. Vehicle doses of DMSO were therefore kept at 0.1% or below where possible but, when necessary 2.5% DMSO can be used as shown above.

3.3.2. Inhibition of CCR5 with the potent CCR5 antagonist maraviroc does not inhibit CCL3 stimulated calcium release in THP-1 cells

Maraviroc has been shown to inhibit chemokine induced signalling in a CCR5 expressing HEK cell line as stimulated by CCL3, CCL4 and CCL5 with an $IC_{50} \leq 30$ nM [11]. Pre-treatment of THP-1 cells with 100 nM maraviroc did not affect the EC_{50} of CCL3 for calcium release, which can be calculated as 25.7 ± 5.4 nM for CCL3 vs. 7.73 ± 0.307 for maraviroc treated cells (Figure 3.3a). Conversely 100 nM maraviroc completely inhibits THP-1 cell migration towards CCL3 (Figure 3.3b.) (unpaired t-test, $p < 0.05$). These data therefore suggest that either CCL3 stimulated calcium release occurs completely via activation of CCR1 or that maraviroc is not a neutral antagonist CCR5 in certain cellular assays.

To further qualify receptor mediated responses, β -chemokine ligand CCL4, which shows high affinity toward CCR5 [42] but no significant interaction with CCR1 in its natural form [222], was used to stimulate calcium release in THP-1 cells.

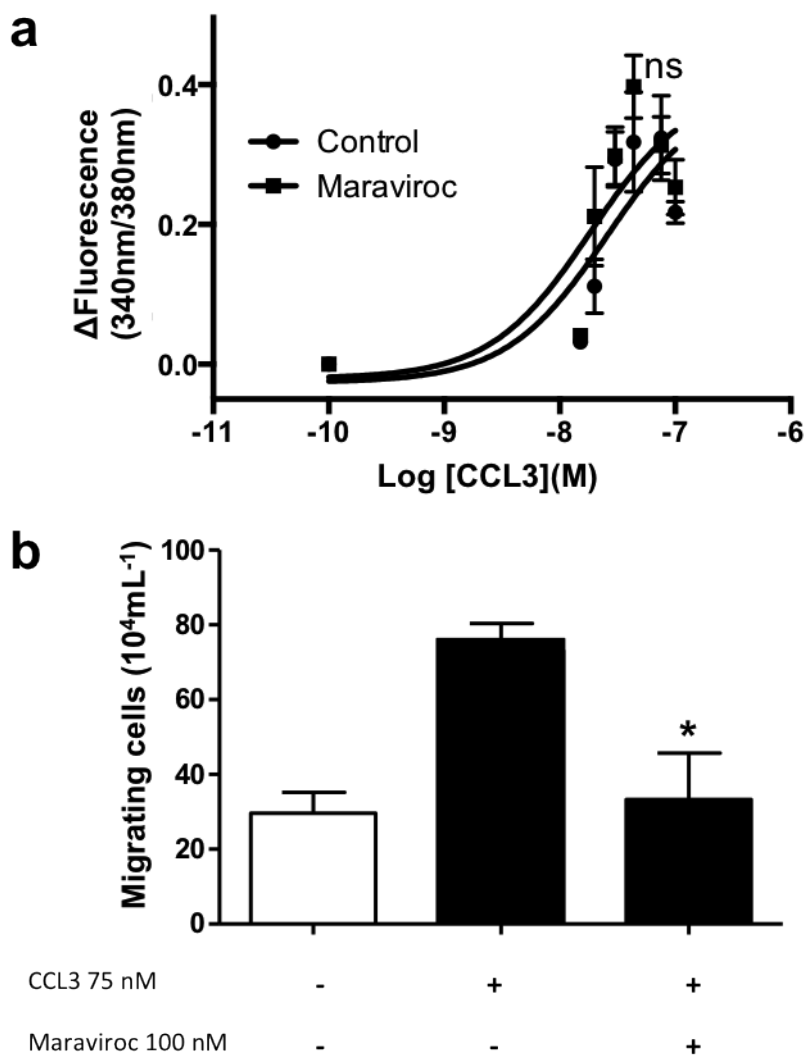


Figure 3.3: CCR5 antagonist maraviroc does not inhibit CCL3 stimulated calcium flux in THP-1 cells but does block migration. (a) Concentration response curves for increasing concentrations of CCL3 stimulating intracellular calcium release in THP-1 treated with 100 nM maraviroc. (b) Maraviroc significantly reduces THP-1 migration towards 1 nM CCL3 (Data from C. Moyano Cardaba). Results represent the mean \pm SEM of at least 3 independent experiments. (Unpaired t-test, ns $p > 0.05$, * $p < 0.05$).

The concentration response for calcium release (Figure 3.4a) shows no marked difference between treatments with the calculated EC_{50} of CCL4 which is 62.7 ± 8.8 nM for untreated vs. 45.6 ± 6.1 nM for maraviroc treated cells. An unpaired t-test showed stimulation at 75 nM CCL4 stimulation was not significantly different between treated and untreated cells. These data

clearly show that CCR5 signalling via CCL4 can stimulate calcium release and also that maraviroc is unable to antagonise CCR5 signalling to calcium release in THP-1 cells.

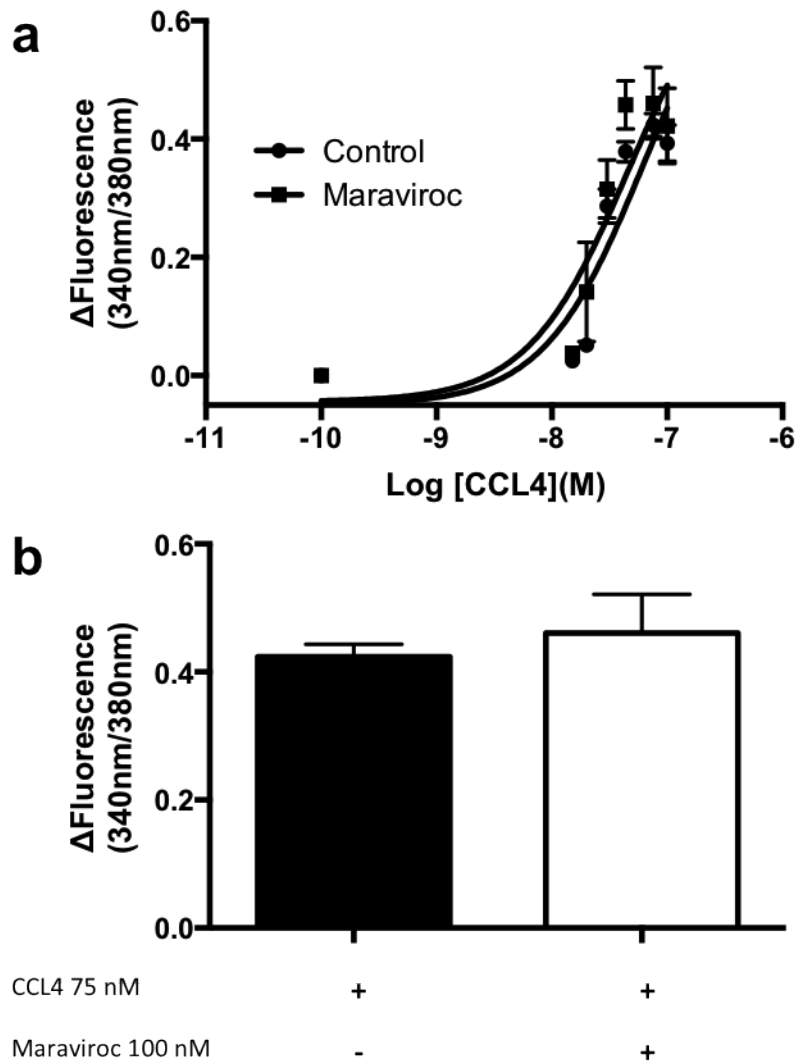


Figure 3.4: CCR5 antagonist maraviroc does not inhibit CCL4 stimulated calcium flux in THP-1 cells. (a) Concentration response curve for increasing concentrations of CCR5 ligand CCL4 stimulating intracellular calcium release in THP-1 cells treated with 100 nM maraviroc. (b) Maraviroc inhibition does not significantly affect THP-1 calcium release stimulated by 75 nM CCL4. Results represent the mean \pm SEM of at least 3 independent experiments. (Unpaired t-test, $p>0.05$).

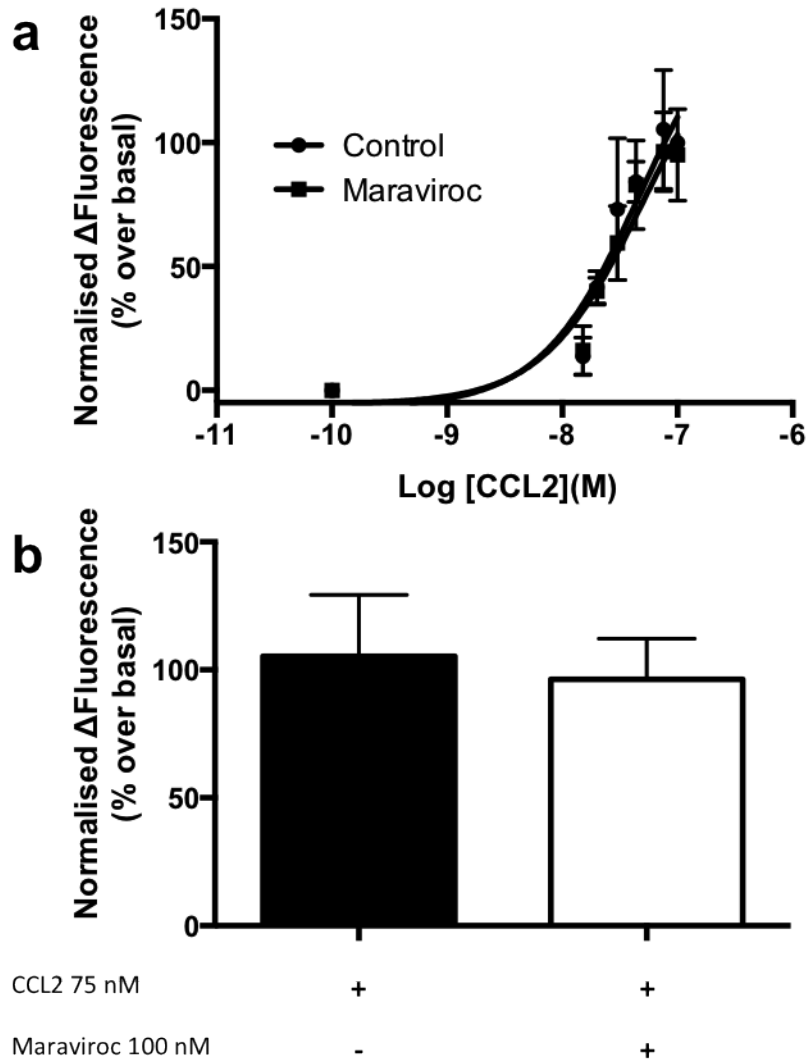


Figure 3.5: CCR5 antagonist maraviroc does not inhibit CCL2 stimulated calcium flux in THP-1 cells. (a) Concentration response curve for increasing concentrations of CCR2 ligand CCL2 stimulating intracellular calcium release in THP-1 cells treated with 100 nM maraviroc. (b) Maraviroc inhibition does not significantly affect THP-1 cell calcium release stimulated by 75 nM CCL2. Results represent the mean \pm SEM of at least 3 independent experiments. (Unpaired t-test, $p > 0.05$).

CCR2 and CCR5 share a high sequence homology compared with other receptors in the CC motif α -chemokine receptor family. Although CCR2 ligand CCL2 shows low activity towards CCR5 [223] and CCR5 ligands show no activity against CCR2; CCR2-CCR5 heterodimerisation and transactivation is well characterised [84]. To determine if CCR5 inhibition had any effect on CCR2 activation in THP-1 cells pre-treated with maraviroc (100 nM) were stimulated with CCL2. It should be noted that CCL2 has been shown to bind to CCR1 [224], however this interaction is unable to induce migration and induces calcium release at 1 μ M CCL2 treatment [225]. For these reasons CCL2 activation of CCR1 is not likely under the test conditions and does not need to be accounted for.

The concentration response for CCL2 stimulated calcium release (Figure 3.5a) was unaffected by maraviroc, with calculated EC_{50} values of 40.3 \pm 9.3 nM for the control vs. 47.2 \pm 4.1 nM for maraviroc treated cells. The level of calcium release stimulated at 75nM CCL2 was also not significantly different (Figure 3.5b) (unpaired t-test $p>0.05$). These data show that CCR2 receptors are present and functional on THP-1 cells and that maraviroc has no effect on their stimulation with CCL2. CCR5 specific signalling was determined by stimulation with CCL4 but maraviroc was unable to affect this interaction. CCL3 stimulated calcium release was also unaffected by maraviroc treatment but because CCL3 also signals through CCR1, the lack of inhibition by maraviroc suggests that CCL3 only acts through CCR1 in THP-1 cells. To test this idea further CCR1 antagonist J113863 was used in conjunction with CCL3 stimulation. The CCR1 inhibitor was used at an excess concentration to ensure that 100% of CCR1 signalling was blocked to ensure that only CCR5 mediated responses were observed.

3.3.3. The potent CCR1 antagonist J113863 significantly inhibits CCL3 stimulated calcium release in THP-1 but does not affect CCL3 EC₅₀

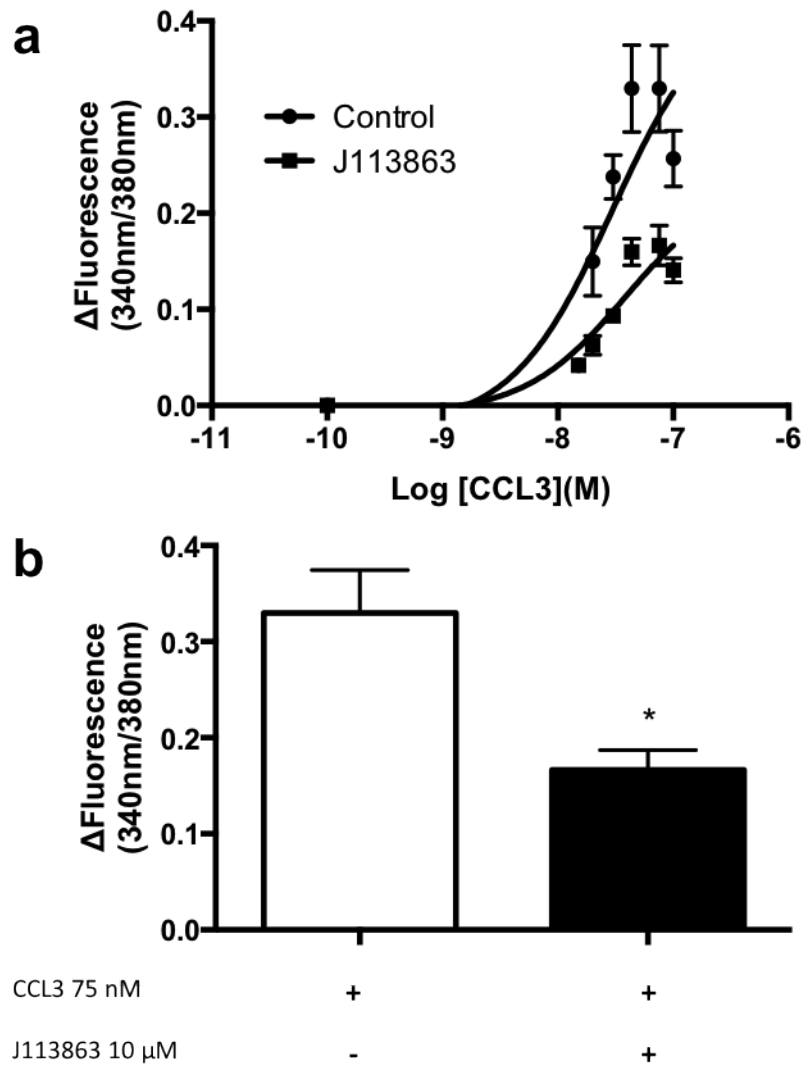


Figure 3.6: CCR1 antagonist J113863 significantly inhibits CCL3 stimulated calcium flux in THP-1 cells. (a) Concentration response curve for increasing concentrations of CCL3 stimulating intracellular calcium release in THP-1 cells treated with 10 μM J113863. (b) J113863 inhibition significantly affects THP-1 cell calcium release stimulated by 75 nM CCL3. Results represent the mean ± SEM of at least 3 independent experiments. (* indicates $p \leq 0.05$ $n \geq 3$ compared to control). (Unpaired t-test, $p > 0.05$).

J113863 is a highly potent low nanomolar IC_{50} antagonist for CCR1 [226] and is inactive against CCR2 and CCR5 [227]. THP-1 cells pre-treated with 10 μ M J113863 showed no significant shift in CCL3 EC_{50} (29.6 \pm 6.7 nM for control vs. 37.8 \pm 8.7 for J113863 treated cells (Figure 3.6a)). Treated THP-1 cells did show a significant reduction in calcium release of \approx 50% (unpaired t-test $p \leq 0.05$ vs. control) (Figure R6b), demonstrating that CCR1 was responsible for at least some signalling to calcium release stimulated by CCL3. The peak CCL3 efficacy for was 46 \pm 10 % lower for J113863 treated THP-1 cells based on predictions from the sigmoidal concentration response curve. The concentration of J113863 used was 1000 times higher than was necessary according to the IC_{50} however, and should have completely inhibited all signalling were CCR1 the only receptor being activated. These data provided evidence that CCL3 was acting through CCR1 (\approx 50%) and another receptor, which the literature and proteomics analysis (Figure 3.1a) suggest might be CCR5.

CCL3 interacts with only CCR1 and CCR5, so treatment of THP-1 cells simultaneously with J113863 and maraviroc should completely inhibit calcium release. THP-1 cells treated with 100nM maraviroc + 10 μ M J113863 show a rise in calcium in response to CCL3 (Figure 3.7a) although at \approx 50% efficacy at 75 nM CCL3 (Figure 3.7b) which represents the same reduction in signalling seen with J113863 treatment alone. These data suggest that either maraviroc potentially is not functioning as an antagonist in this system or it could be reflection of agonistic behaviour of maraviroc towards CCR5 or J113863 could act as an agonist for CCR5.

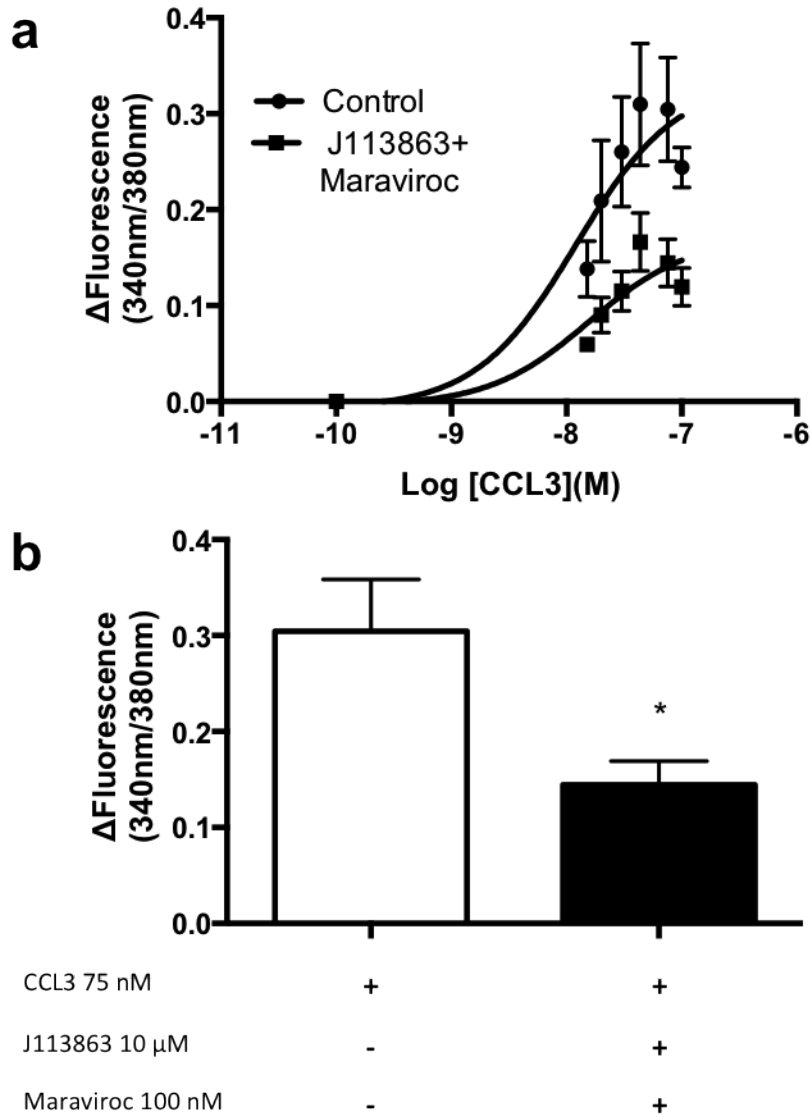


Figure 3.7: A combination of maraviroc and J113863 significantly reduces CCL3 stimulated calcium release in THP-1 cells. a) Concentration response curve for increasing concentrations of CCL3 stimulating intracellular calcium release in THP-1 treated with both 10 μM J113863 (J113) & 100 nM maraviroc (Mara). (b) J113+Mara inhibition significantly affects THP-1 calcium release stimulated by 75 nM CCL3. Results represent the mean ± SEM of at least 3 independent experiments. (Unpaired t-test, $p > 0.05$. * Indicates $p \leq 0.05$ $n \geq 3$ compared to control).

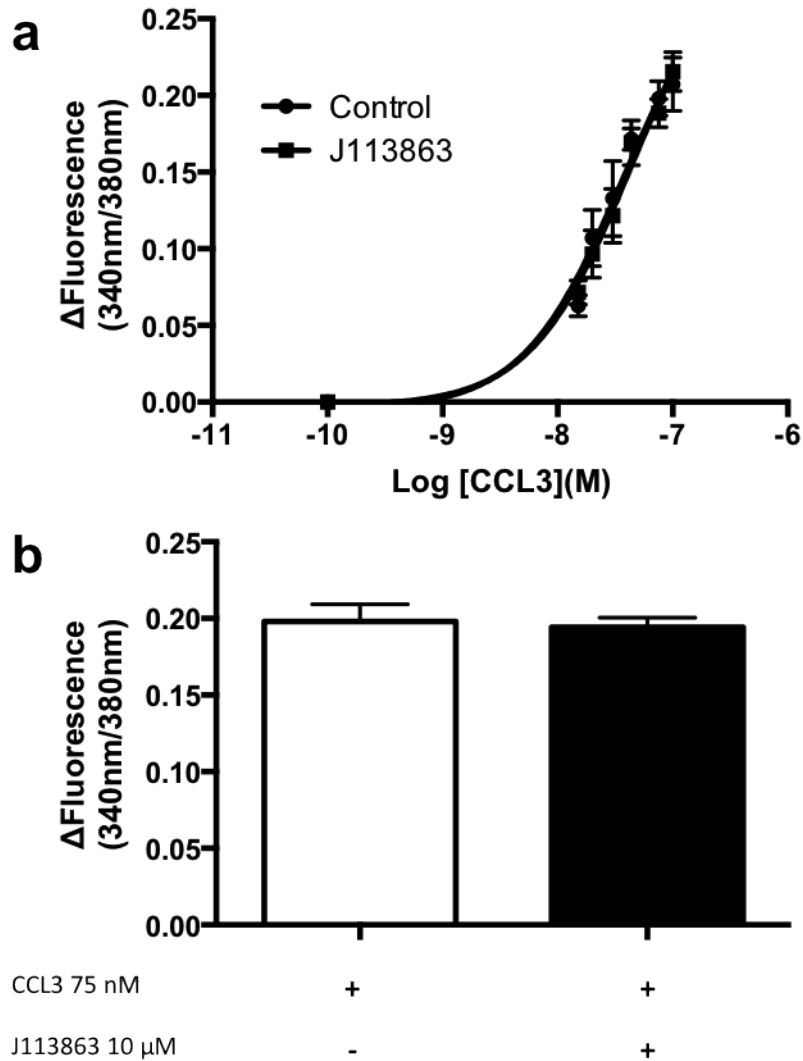


Figure 3.8: CCR1 antagonist J113863 does not inhibit CCL3 stimulated calcium flux in HeLa.RC49. (a) Concentration response curve for increasing concentrations of CCL3 stimulating intracellular calcium release in HeLa.RC49 treated with 10 μM J113863 (J113). (b) J113 inhibition does not significantly affect calcium release stimulated by 75 nM CCL3 compared with untreated control. Results represent the mean ± SEM of at least 3 independent experiments. (Unpaired t-test, $p > 0.05$).

The adherent cell line HeLa.RC49 is stably transfected with CCR5 and shows high levels of receptor expression (Figure 3.1a) and, as the cells are fibroblasts, negligible CCR1 expression was expected. Treatment of HeLa.RC49 with 10 μM J113863 did not alter the EC_{50} of calcium release significantly for CCL3 induced signalling nor did it affect the response at 75 nM for cells

stimulated with CCL3 (Figure 3.8). These data clearly indicate that CCL3 can stimulate calcium release via CCR5 but also that J113863 does not affect CCL3 mediated signalling through CCR5 indirectly.

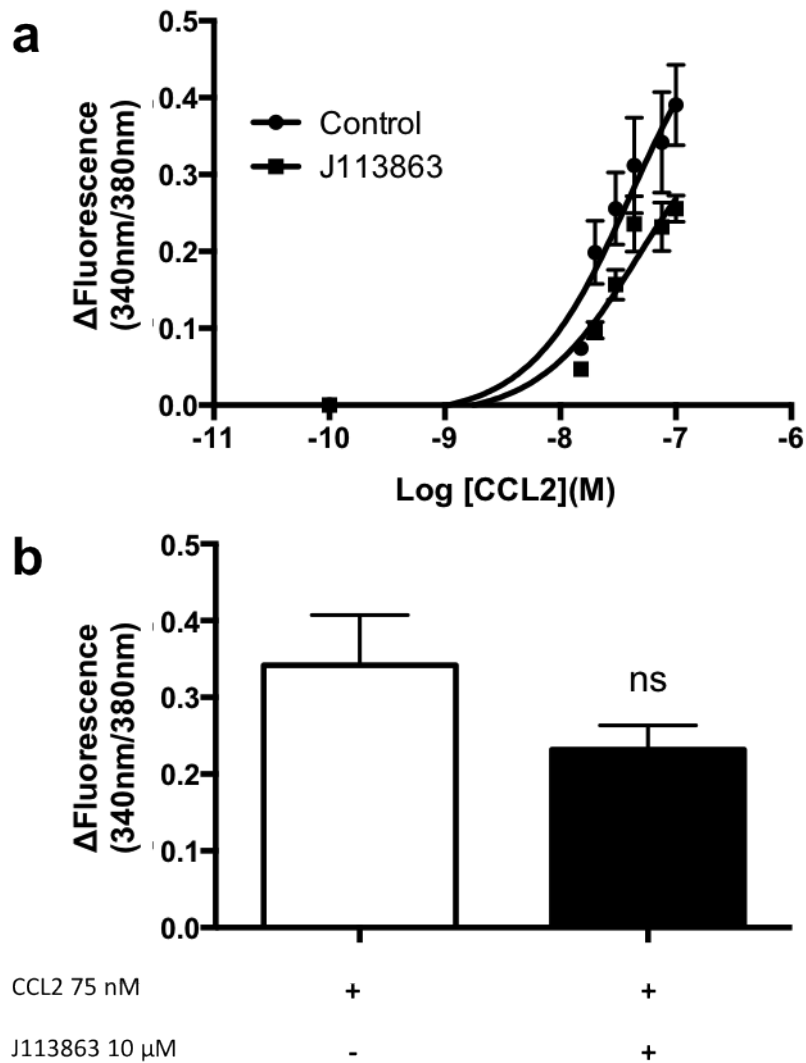


Figure 3.9: CCR1 antagonist J113863 does not inhibit CCL2 stimulated calcium flux in THP-1 cells. (a) Concentration response curve for increasing concentrations of CCR2 ligand CCL2 stimulating intracellular calcium release in THP-1 cells treated for 0.5hr with 10 μM J113863 prior to assay. (b) J113863 inhibition does not significantly affect THP-1 cellular calcium release stimulated by 75 nM CCL2. Results represent the mean ± SEM of at least 3 independent experiments. (Unpaired t-test. ns = not significant p>0.05, n=3).

J113863 did not significantly affect 'non-target' chemokine receptor signalling in HeLa.RC49 cells but to determine whether this observation was true in THP-1 cells the effect of pre-treatment with J113863 (10 μ M) on CCL2 stimulated calcium release was determined. Pre-treated cells showed a non-significant (unpaired t-test $p>0.05$) trend towards decreased signalling stimulated by CCL2 (Figure 3.9). Whilst this trend may be explained by the inhibition of CCR1-CCR2 heterodimer signalling a simpler explanation is that some cytotoxicity or non-specific disruption is being induced by high inhibitor concentrations. This is supported by the fact that THP-1 cells are known to be less resilient than the immortalised adherent fibroblasts such as HeLa and CHO cells [228]. The non-significant decrease in CCR2 stimulated calcium release was also too low to explain the results seen with CCL3 stimulation (Figure 3.6.) suggesting these observations were due to inhibition of CCR1.

3.3.4. Maraviroc stimulates calcium release in THP-1 cells

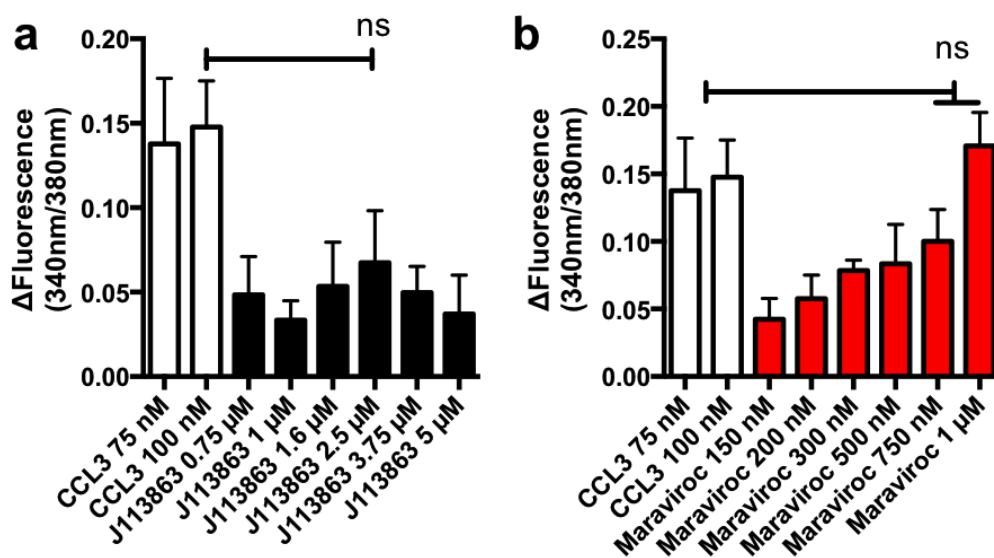


Figure 3.10: Maraviroc behaves as an agonist in THP-1 cell calcium flux assay by stimulating calcium release. (a) Stimulation of THP-1 cells with CCR1 antagonist J113863 has no concentration dependent effect on THP-1 intracellular calcium release. (b) Stimulation with maraviroc (M) stimulates intracellular calcium flux in a concentration dependent fashion with 300-1000 nM stimulation not significantly different from 75 nM CCL3 (One-way ANOVA, Bonferroni multiple comparison. $p > 0.05$ $n = 4$). Results represent the mean \pm SEM of at least 3 independent experiments.

Because J113863 was shown not to be interfering with non-specific receptor activation the remaining explanation for the observed lack of CCR5 signalling inhibition was that maraviroc was stimulating calcium release in THP-1 cells. To test this hypothesis, THP-1 cells were stimulated with increasing concentrations of J113863 and maraviroc (Figure 3.10) and the resulting calcium flux was compared with control cells stimulated only with 75 or 100 nM CCL3, respectively. Although J113863 had a minor effect on calcium release, the ratio of fluorescence intensity remained significantly lower than that seen with the 75 nM CCL3 in control cells and did not show any kind of concentration dependent relationship. Maraviroc, on the other hand, was able to stimulate calcium release in THP-1 cells; stimulation with concentrations of

maraviroc of 300 nM and above induced a concentration dependent calcium release that was not significantly lower than that achieved with 75nM CCL3 in control cells. These data indicate that the allosteric CCR5 inhibitor maraviroc acts as an agonist on THP-1 cells for calcium release. However, whether this is via direct stimulation of CCR5 or via a 'non-specific' cell type dependent mechanism cannot be said. It has been shown that maraviroc can successfully inhibit CCR5 mediated calcium release in other cell lines [11] so the latter may be the better explanation. CCR5 signalling has been shown to be function differently in THP-1 cells than in adherent fibroblasts particularly in response to alterations in membranes fluidity. Cell type specific differences in CCR5 signalling may explain the effect of maraviroc but this does not change the fact that the inhibitor is not suitable for use in THP-1 where calcium release is to be measured.

3.3.5. Analysis of CC motif chemokine receptor activation and chemotaxis in THP-1 cells

The experimentation with CCL3 induced calcium release revealed an unexpected agonistic effect of maraviroc at high antagonist concentration. There are reasons to believe that the effect of maraviroc may be limited to calcium release in THP-1 cells and the inhibitor may still be of use in clarifying chemokine receptor activated chemotaxis. Firstly our research group has already shown that 100 nM inhibits THP-1 chemotaxis towards 1 nM CCL3 (unpublished observation) and secondly CCR5 mediated calcium release and chemotaxis are independent in THP-1 cells as shown by Cardaba *et al.* [75]. With these facts in mind it was important to determine if CCL3 mediated chemotaxis could be attributed to a specific receptor and if the model system could be modulated to isolate CCR5 specific responses with Maraviroc and J113863.

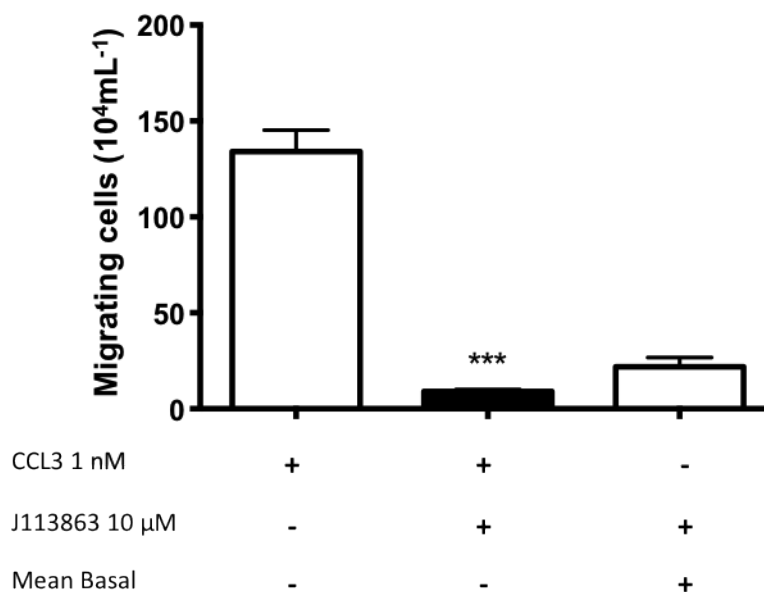


Figure 3.11: Inhibition of CCR1 reduces THP-1 cell migration towards 1 nM CCL3 to sub-basal levels. 0.5hr Pre-treatment of THP-1 cells with 10 μM J113863 significantly reduces THP-1 chemotaxis stimulated by CCL3 to sub basal levels (One-way ANOVA, Bonferroni multiple comparison. * = $p \leq 0.001$ $n \geq 3$). Results represent the mean \pm SEM of at least 3 independent experiments.**

Treatment of THP-1 cells with 10 μM J113863 was used to determine the role of CCR1 in chemotaxis stimulated by 1 nM CCL3 (Figure 3.11). Chemotaxis was significantly reduced to below unstimulated (basal) levels (one way ANOVA followed by Bonferroni multiple comparison, $p \leq 0.001$ vs. stimulated vehicle control). Stimulated control cells migrated significantly higher than unstimulated control ($p \leq 0.001$) but a distinct trend towards reduction was also observed in basal migration of cells treated with J113863 compared with vehicle controls although this was deemed to be not significant ($p > 0.05$). Considering that chemokine receptor agonists target the extracellular membrane and would not be expected to interfere with basal migration this came as a surprise and indicated that the inhibitor maybe disrupting migration in a non-specific fashion as postulated for CCL2 stimulated calcium release (Figure 3.9). To test this hypothesis THP-1 cell migration was tested against decreasing concentrations of J113863.

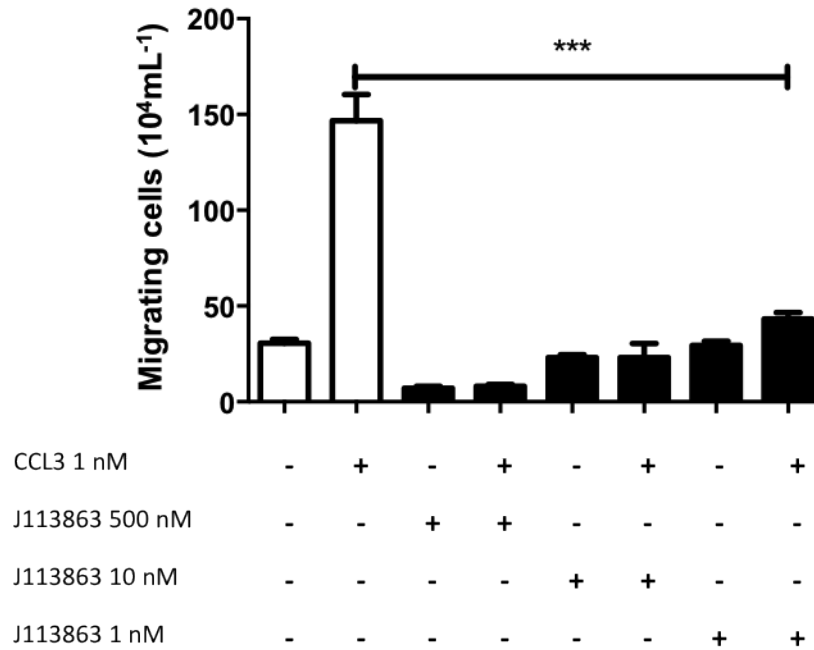


Figure 3.12: J113863 significantly inhibits THP-1 cell chemotaxis towards 1 nM CCL3 at concentrations down to 1 nM. THP-1 cells pre-treated for 0.5hr with J113863 at 500, 10 and 1 nM all show significant inhibition of migration down to and below basal levels. Results represent the mean \pm SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison. *Indicates $p \leq 0.001$ vs. vehicle $n \geq 3$).**

THP-1 pre-treated with 500, 10 and 1 nM J113863 showed a significant reduction in migration ($p \leq 0.001$) compared to vehicle control (Figure 3.12). At 500 nM basal migration of treated cells was still lower than vehicle basal but basal treated migration was restored to control levels at 10 and 1 nM treatments. Surprisingly the reduction in chemotaxis towards CCL3 at 1 nM J113863, which represents the calculated IC_{50} [227], was not significantly different from vehicle basal migration ($p > 0.05$). To determine if the effect of J113863 treatment was specific to CCL3 stimulation THP-1 cells treated the same decreasing concentrations of J113863 were stimulated in chemotaxis assay with 1 nM CCL4.

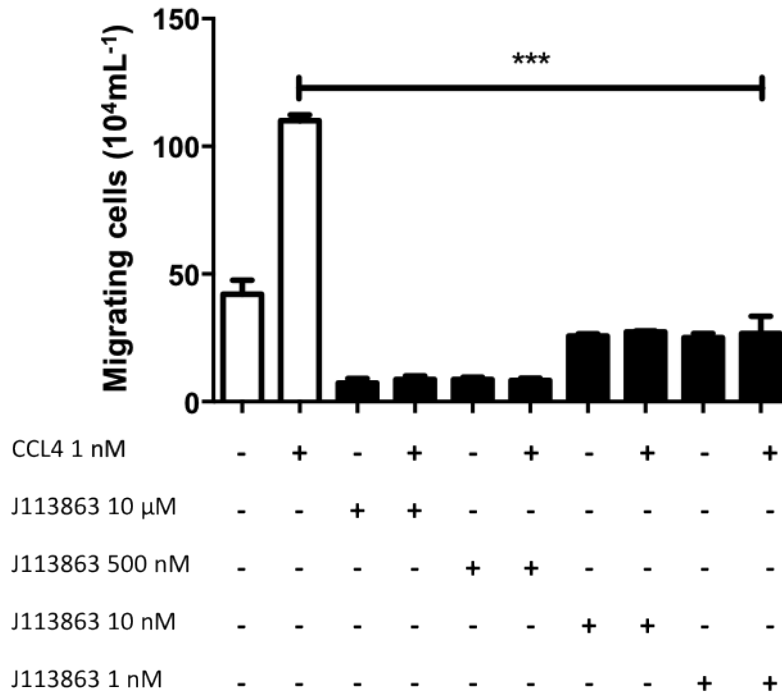


Figure 3.13: J113863 significantly inhibits THP-1 cell chemotaxis towards 1 nM CCL4 at concentrations down to 1 nM. THP-1 cells pre-treated for 0.5hr with J113863 at 10 μM, 500, 10 and 1 nM all show significant inhibition of migration down to and below basal levels. Results represent the mean ± SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison.*Indicates p≤0.001 vs. vehicle n≥3).**

As with CCL3 stimulation the four pre-treatment concentrations of J113863 (10 μM, 500, 10 and 1 nM) all significantly reduced THP-1 chemotaxis (Figure 3.13) (p≤0.001, n≥3). The level of reduction at 10 μM and 500 nM was significantly lower than basal migration in untreated cells and basal migration in treated cell was also significantly lower than control basal (p≤0.001). As with CCL3 stimulation, a recovery of basal migration occurred at 10 and 1 nM J113863 treatment (p>0.05 vs. control basal). This was surprising considering J113863 had no significant effect on CCL4 stimulated calcium release and strongly suggested that ‘off target’ effects were responsible for the observed reduction in migration.

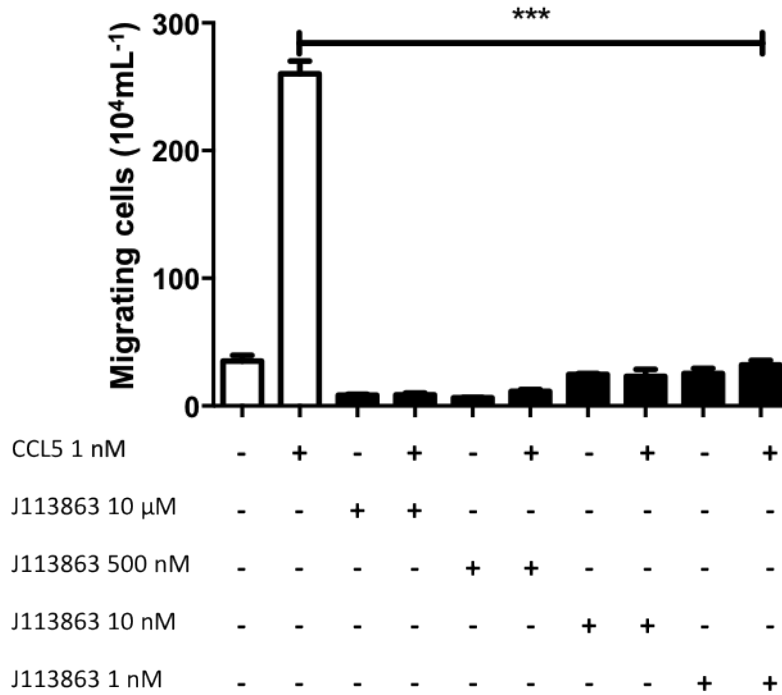


Figure 3.14: J113863 significantly inhibits THP-1 cell chemotaxis towards 1 nM CCL5 at concentrations down to 1 nM. THP-1 cells pre-treated for 0.5hr with J113863 at 10 μM, 500, 10 and 1 nM all show significant inhibition of migration towards 1 nm CCL5 down to and below basal levels. Results represent the mean ± SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison. *Indicates $p \leq 0.001$ vs. vehicle).**

CCL5 stimulation was also used to determine if the effect of J113864 could be abrogated. 1 nM CCL5 stimulated a greater control migration than that seen with either CCL3 or CCL4 but the same pattern in inhibition was seen with the four concentration treatments of J113863. All treatments significantly reduced migration to that of control basal or below ($p < 0.01$ vs. stimulated control) with 10 and 1 nM treatments showing control migration restored to unstimulated control levels (Figure 3.14).

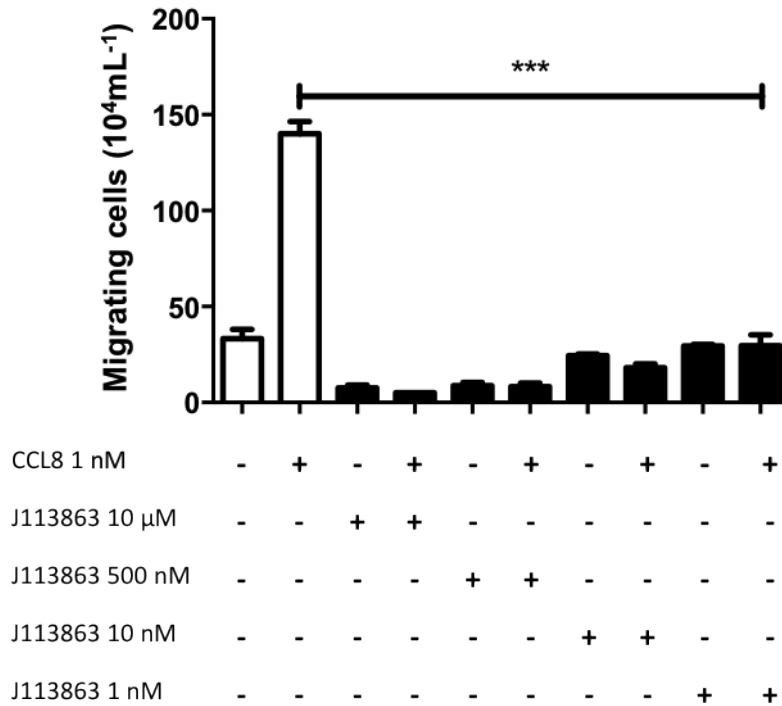


Figure 3.15: J113863 significantly inhibits THP-1 cell chemotaxis towards 1 nM CCL8 at concentrations down to 1 nM. THP-1 cell pre-treated for 0.5hr with J113863 at 10 μM, 500, 10 and 1 nM all show significant inhibition of migration down to and below basal levels. Results represent the mean ± SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison. *Indicates p≤0.001 vs. vehicle).**

CCL8 interacts with CCR1, 2 and 5 [5] and THP-1 cells migrate towards 1 nM CCL8 strongly [47] (Figure 3.15). Despite being able to interact with three chemokine receptors known to be expressed on THP-1, J113863 at the four pre-treatment concentrations significantly inhibited stimulated migration (p≤0.001, n≥3) and reduced non stimulated migration to levels significantly lower than control basal at 10 μM and 500 nM (p≤0.01).

These combined data provide support for the idea that J113863 disrupts migration in THP-1 cells in an ‘off target’ fashion; the three classical CCR5 agonists CCL3/4/5 have differing affinity for CCR5 and CCR1 but show similar responses to J113863 inhibition which seems unfeasible unless all signalling was occurring via CCR1 alone. This hypothesis also does not fit the data: firstly CCL4 does not interact with CCR1 and secondly the J113863 IC₅₀ for CCR1 inhibition is

around 1 nM. If the effect of J113863 was wholly through inhibition of CCR1 then the expected inhibition at 1 nM would be significantly lower than the complete inhibition observed. The effects of J113863 on calcium release also do support this idea, as 10 μ M J113863 only reduces calcium release by \approx 50%, the remainder of release occurring via CCR5 activation. This suggests CCR5 is being activated by CCL3 and the activation of CCR5 is known to stimulate to migration [29]. It is clear that the effects of J113863 on migration are not related to its ability to inhibit CCR1, but the data do not provide adequate information to clarify the mechanism by which this additional inhibition occurs. HeLa.RC49 pre-treated with 10 μ M J113863 showed no abnormalities in actin polymerisation (data not shown), which eliminates disruption of actin fibre formation as an explanation.

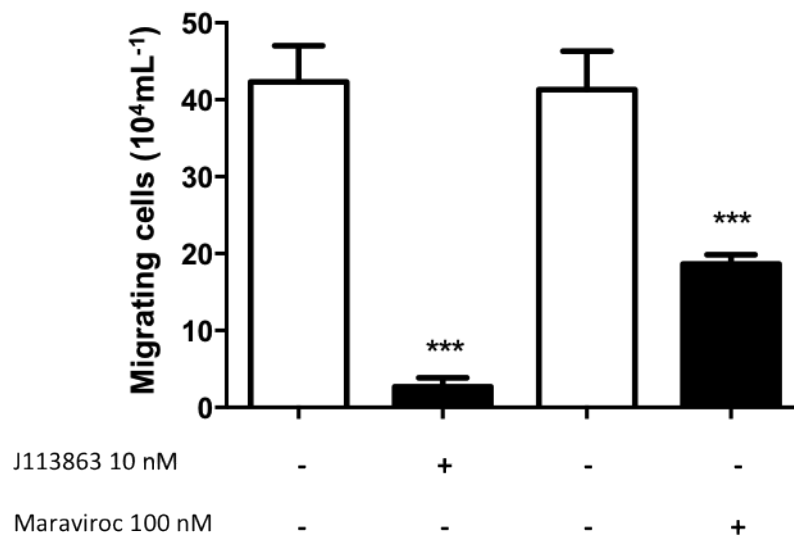


Figure 3.16: J113863 and maraviroc do not stimulate chemotaxis in THP-1 cells, but block basal level of migration in the absence of chemokine. Untreated THP-1 cells showed levels of migration significantly lower than basal towards either 10 μ M J113863 or 100 nM maraviroc ($p \leq 0.001$ vs. basal $n \geq 3$). Results represent the mean \pm SEM of at least 3 independent experiments (Unpaired t-test, * indicated $p \leq 0.001$ vs. basal control $n \geq 3$).**

The observations would be explained if J113863 acted as an agonist and stimulated migration. If this was the case, then treatment of THP-1 cells with J113863 prior to a chemotaxis assay would be tantamount to placing chemoattractant in apical and basal wells of the transwell migration assay therefore eliminating any gradient towards which cell could migrate. To test this idea untreated THP-1 cells were stimulated in a transwell migration assay with 10 nM J113863 or 100 nM maraviroc (in the basal chamber). Both J113863 and maraviroc significantly reduced basal migration, i.e. migration not in response to a chemokine, (Figure 3.16) of THP-1 cells ($p \leq 0.01$ vs. non treated cells). This indicates not only that the inhibitors do not stimulate migration but also that basal migration is reduced in their presence. This additionally provides clear evidence that maraviroc does not behave as a CCR5 agonist in chemotaxis assay.

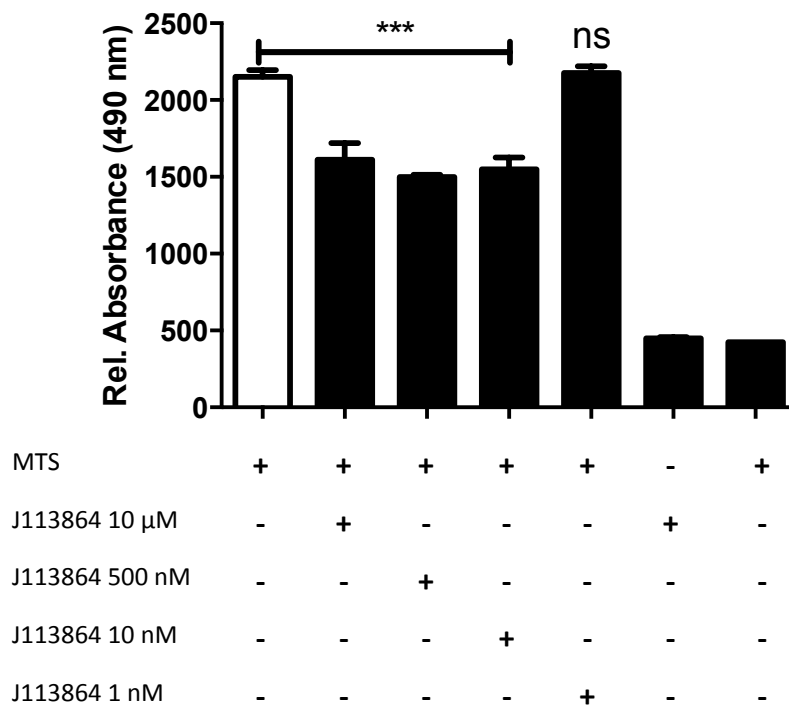


Figure 3.17: J113863 affects THP-1 cell viability after 5hr incubation. Pre-treatment of THP-1 with varying concentrations of J113863 followed by 5hr incubation with MTS reveals a significant reduction on MTS metabolism for cells treated with 10 μM and 500 and 10 nm J113 (n=3). Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.001$ vs. untreated control).**

An alternate explanation to the observed effects of J113863 treatment on THP-1 cell migration could be cytotoxic effects. The colourimetric (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) metabolism assay was used to determine any cytotoxic effects on THP-1 cells treated with 10 μ M, 500, 10 and 1 nM for 0.5hr and then incubated with MTS for a further 5hr (described in material and methods). (Figure 3.17). J113863 at 10 μ M, 500 and 10 nM significantly reduced MTS metabolism ($p \leq 0.001$) over 5hrs however MTS metabolism was also significantly higher than in the negative control ($p \leq 0.001$). This reduction in viability was not concentration dependent and 1 nM J113863 showed no significant cytotoxicity vs. the vehicle control, which means that any cytotoxic effects of J113863 do little to explain the observed reductions in migration in THP-1 cells.

3.3.6. THP-1 cells do not migrate towards α chemokine ligand CXCL12

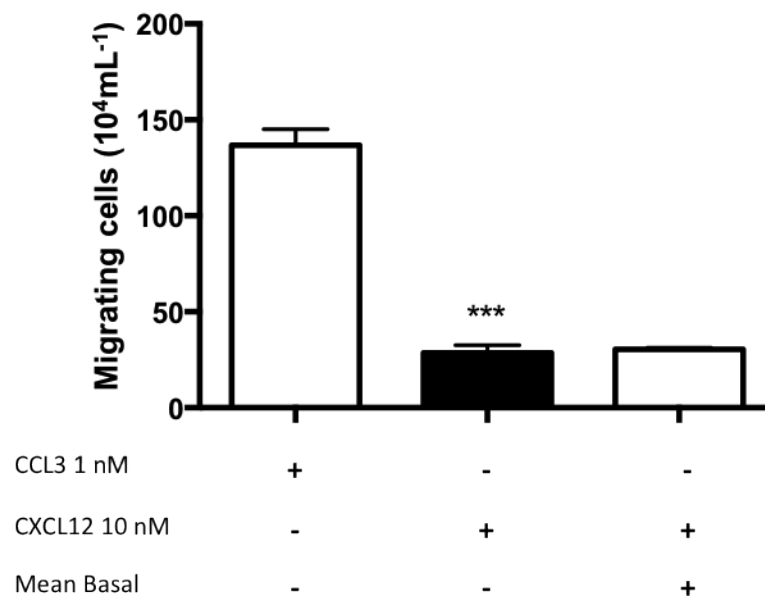


Figure 3.18: THP-1 cells do not migrate towards CXCR4 agonist CXCL12. Stimulation of untreated THP-1 cells with 10 nM CXCL12 showed no significant migration (n=3). Results represent the mean \pm SEM of 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison. *** = $p \leq 0.001$.)

It has been shown that CXCR4 is expressed on THP-1 cells [134], however in our hands THP-1 cells did not migrate towards 10 nM CXCL12 (Figure 3.18), a concentration widely used to promote chemotaxis towards CXCL12. This data may represent absence of CXCR4 in the subpopulation of THP-1 or a failure of activated cell to transduce signalling into chemotactic machinery. In either case the inability of cells to undergo migration towards CXC-motif chemokines (Figure 6.37) simplifies the analysis of the signalling events observed in the cell line.

3.4. Discussion

The aim of this chapter was to determine if THP-1 cellular responses could be modulated with small molecule chemokine receptor antagonists, so that conclusions about CCR5 specific signalling could be made. This experimentation was required because chemokine ligands can stimulate numerous receptors and activated receptors are susceptible to signalling cross talk and transactivation [84]. Therefore it seemed paramount to start with an overview of the different cellular responses in THP-1 cells and use small molecule inhibitors to differentiate between signal transduction pathways of different chemokine receptors, aiming at isolating the CCR5 specific responses in these cells. The importance of determining receptor signalling through functional responses as opposed to relying on proteomics or the literature is exemplified by the response of THP-1 cells to CXCL12. Although CXCR4 is widely shown to be expressed on THP-1 in the literature [134], no significant migratory response was observed with the THP-1 cell population used in the assays here. This shows that in this system no conclusions can be drawn about the function role of CXCR4 regardless of whether or not they are expressed.

The experimental data concerning C-C motif chemokine receptor mediated responses in THP-1 cells presents a conflicting picture when taken at face value. THP-1 calcium release can be stimulated by CCL2, CCL3 and CCL4 which correspond to the chemokine receptors CCR1, CCR2 and CCR5 as identified in the literature [178, 216-219]. Maraviroc treatment, however, was unable to inhibit CCL3 stimulated calcium fluxes which suggested that CCL3 was acting predominantly through CCR1. The failure of maraviroc to inhibit the CCR5 specific ligand CCL4

provided contrary evidence and indicated that the inhibitor was either not functioning or was stimulating calcium release. Maraviroc is well characterised as an inhibitor of CCR5 mediated signalling in CCR5 expressing fibroblasts [11], so these observations came as a surprise. The potent CCR1 inhibitor J113863, which shows no activity against CCR2 and CCR5 [227] was used to examine the nature of CCL3 stimulated calcium release. J113863 was used at excess so that inhibition of CCR1 mediated response was certain. Treatment with J113863 at this concentration reduced calcium release by $\approx 50\%$ indicating that CCL3 induced calcium release was mediated by CCR1 and CCR5 in approximately equal proportions, at least in terms of cellular response. These data supported the idea that J113863 could be used to force signalling through CCR5 so that signalling pathways specific to the receptor could be isolated. The failure of maraviroc to affect CCR2, 3 and 4 stimulated calcium release can be explained by the fact that stimulation of THP-1 with maraviroc lead to a concentration dependent calcium release. Whilst the concentrations of maraviroc that induced the largest response ($1 \mu\text{M}$) was higher than that used to inhibit CCR5 (100 nM), the exposure time to stimulants in calcium flux assay is considerably shorter than the 0.5hr incubation time used for inhibition. We can hypothesise that prolonged exposure to a lower level of maraviroc would disrupt calcium signalling and mask any inhibitory effects that may have arisen from the inhibition of CCR5 agonist binding. Whilst the data do not provide sufficient information to form definite conclusions about the nature of this stimulation beyond speculation, they do show that maraviroc cannot be used with THP-1 where calcium release is to be determined. This did not rule maraviroc out as a useful tool for manipulating chemokine receptor signalling because chemotaxis in THP-1 has been shown to be independent of calcium release [75] and sensitive to maraviroc inhibition.

Despite J113863 behaving as expected in THP-1 cell calcium flux assays, treatment of cells with the inhibitor prior to chemotaxis assays resulted in inhibition of CCL3, 4, 5 and 8 stimulated migration which was not consistent with CCR1 inhibition alone. The effect of J113863 on THP-1 chemotaxis suggested that the inhibitor was altering the ability to migrate in an unspecific fashion by virtue of the fact that chemotaxis in treated cells was reduced to sub-basal levels (Chapter 3.3.5). Although CCR1 is known to be expressed in THP-1 cells, direct proteomic

identification of the receptor was not carried out in this sub-population, however, as with the results with CXCR4, the aim of this chapter was to outline the functional receptor profile for THP-1 so whether any of the effects of J11386 can be considered 'on-target' are irrelevant.

Cytotoxic effects, which were shown to be significant at higher J113863 concentrations, may explain some of these observations but provide no answers as to why 1 nM J113863 is so effective at blocking chemotaxis when the calculated IC_{50} value for inhibition \approx 1 nM and therefore the antagonist should not be able to completely block migration at this concentration [227]. Due to these observations the use of J113863 in future assays to promote CCR5 mediated responses cannot be reasonably considered, as it will add variables that are not fully accounted for and which may alter signalling responses of CCR1 and CCR5 in unpredictable ways. Other CCR1 [229, 230] and CCR5 [10] antagonists are available, but as both J113863 and maraviroc have displayed unspecific effects, any potential chemokine inhibitor must also be carefully characterised to determine any irregularity before use. As the introduction of variables that have unpredictable effects on signal transduction is clearly more detrimental than having more poorly resolved but predictable signalling chemokine receptor antagonist will not be used in future assays.

This still leaves the question of how to attribute cellular responses stimulated by chemokines that are known to interact with both CCR1 and CCR5. Protein knock down via siRNA could be employed to prevent CCR1 expression but this then adds a range of other variables to be accounted for not to mention the increase in time needed for simple assays. THP-1 cells are known as a 'difficult to transfect' cell line [228], so adding additional experimental stages, including the associated exposure to chemical vehicles and electroporation etc. are likely to decrease the resolution with which signalling events can be determined. A simpler solution, based on the experimental data from this chapter, would be to introduce the caveat that from here forth CCL3 stimulated responses though CCR1 and CCR5 will be called CCR5 mediated responses. HeLa.RC49 cells (which highly express CCR5 and not CCR1) and CHO.CCR5 cells (which only express the human receptor CCR5) can be used to cross analyse the results. Any

discrepancies in the observations seen can then be attributed in part to CCR1 activity in THP-1. In the worst case scenario observations are being made from two highly homologous receptors of the same family [231]: J113863 and maraviroc did not affect CCR2 signalling and there was not migratory response to the α -chemokine ligand CXCL12 (and CXCL11 see later chapter). If conclusions drawn using this approach are unclear then receptor knockdown with siRNA can be used to resolve CCR5 specific mediation from CCR1. The simplicity of the proposed solution is likely to allow a greater amount of experimentation to be obtained per unit time without adversely affecting the sensitivity or reliability of the assays used.

3.5. Chapter Conclusions

The final conclusions to be drawn from this chapter are:

- 1) Chemokine receptors CCR1, CCR2 and CCR5 are expressed by THP-1 cells and can induce cellular responses through the binding of chemokine ligands.
- 2) CXCR4 is not present or cannot induce migration in THP-1 cells
- 3) The chemokine receptor antagonists maraviroc and J113863 cannot be used to block specific chemokine receptor activation in THP-1 because they have non-specific effects which unpredictably affect cellular responses.
- 4) To reduce the likelihood of these unpredictable effects resulting in erroneous conclusion regarding the nature of CCR5 signal transduction, CCR5 specific signalling will be determined by cross analysis of responses in CCR5 positive, stably transfected cell lines which do not express other chemokine receptors.

CHAPTER 4: $G\alpha$, $G\beta\gamma$ or arrestin: Resolution of the initial signal transduction route to chemotaxis

4.1. Introduction

CCR5 is a GPCR and a β -chemokine receptor and as such there are many possible routes describes in the literature by which receptor activation could lead to signal transduction which results in the relevant cytoskeletal rearrangements necessary for migration. Even signal transduction events specific to CCR5 seem contradictory [29, 39, 43, 115, 232] and determining whether these observations are receptor or cell type specific is not possible from the literature alone. There are also unanswered questions about the role of signalling proteins known to be involved in CCR5 mediated signal transduction and their role in migration, such as arrestin scaffolds, which have been shown to form in numerous different ways [35, 39, 65] and non-receptor tyrosine kinases which have been shown to be involved with $G\alpha_i$ signalling [29, 96] and arrestin scaffolds [35, 39]. For a more in-depth dissection of chemotaxis in THP-1 cells to be possible it will be important to identify key points in signal transduction. These points should allow comparisons to be made with CCR5 specific events identified in the literature so that any conflicting information could be brought into context, ultimately allowing a clearer picture of the signal transduction to arise. Conclusions drawn from this chapter will allow further investigation into migration to be targeted towards specific and pertinent protein interactions.

4.2. Chapter Aims

The primary aim of this chapter is to identify by proteomic and inhibitory means the 'route' by which activated CCR5 receptors transduce signals to chemotaxis. These techniques will be used against $G\beta\gamma$, arrestin and arrestin recruitment kinases which should allow the initial route of

signal to be clarified. This will allow future observations to be related to a specific signalling axis and allow comparison to the relevant literature.

4.3. Results

4.3.1: G $\beta\gamma$ is not required for CCL3 stimulated THP-1 cell migration

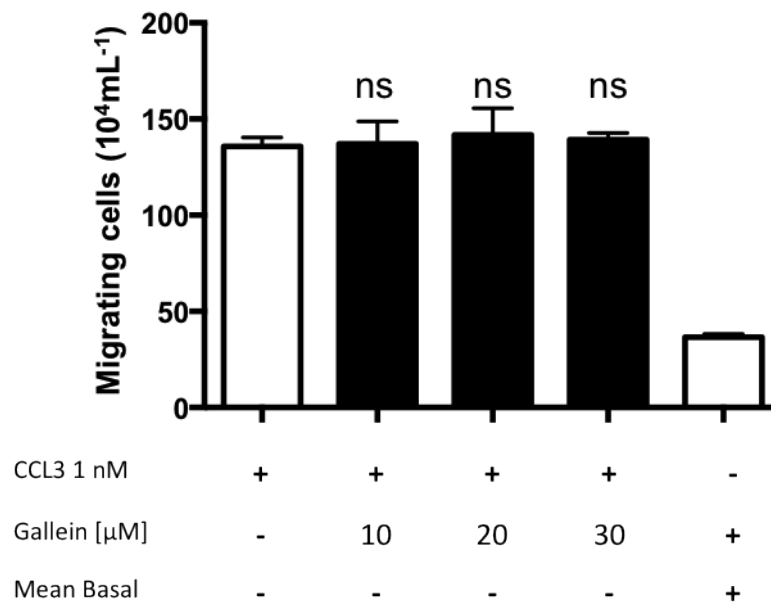


Figure 4.1: G $\beta\gamma$ inhibitor gallein has no significant effect on THP-1 migration towards CCL3. Pre-treatment for 0.5hr with 10, 20 or 30 μM gallein does not significantly alter THP-1 chemotaxis towards 1 nM CCL3 vs. vehicle control. All results represent the mean \pm SEM of 6 independent experiments (One-way ANOVA, Bonferroni multiple comparison, ns = not significant).

As discussed in the introduction GPCR signalling to chemotaxis is canonically associated with G $\beta\gamma$ signalling through PI3K, PKB and MAPKs [55, 57]. G $\beta\gamma$ interaction with GRK2 is also a documented route for transduction to signalling [53]. Protein knock down of G $\beta\gamma$ subunits is difficult due to the large number of isotypes present in cells and the redundancy in signalling seen in G $\beta\gamma$ dependent axes. Therefore the small molecule inhibitor of G $\beta\gamma$, gallein, [57] was used to investigate the role of the protein heterodimer in CCR5 mediated chemotaxis. Gallein binds to the G $\beta\gamma$ binding 'hotspot' preventing interactions with PI3K and GRK2 and has been shown to inhibit fMLP stimulated chemotaxis in HL60 cells which supports the canonical view of G $\beta\gamma$ signalling in GPCR mediated migration [57]. THP-1 cells were pre-treated with 10, 20 and 30 μ M gallein for 0.5hr prior to stimulation with 1 nM CCL3 in a transwell migration assay (Figure 4.1). Gallein was unable to significantly affect THP-1 cell migration towards CCL3 at the three treatment concentrations ($p > 0.05$ vs. vehicle control, $n=6$). These data suggest that CCR5 chemotaxis is not G $\beta\gamma$ dependent. Gallein was used up to 1.2 mM pre-treatment concentrations (data not shown) with no affect to CCR5 migration.

It was important to determine the relevance of these finding in primary tissue. Whilst THP-1 are validated as models for leukaemia and chronic inflammatory disease, there is the possibility that abnormalities in signalling in the immortalised cancer cell line are producing signal transduction pathways with no relevance to normal human tissue. To validate the findings in THP-1 cells IL-2 and concanavalin A activated peripheral blood lymphocytes (PBLs), which have been shown to express CXCR3 and CCR5 upon activation [32], were used. PBLs are derived from adherence depleted Peripheral Blood Mononuclear Cells (PBMCs) and represent a population of T-cells, B-cells and Natural Killer cells. Activation with IL-2 and concanavalin A (Con A) stimulates the upregulation of CXCR3 and CCR5 on T-cells [32], so migratory responses seen in PBLs towards CXCL11 and CCR5 ligands are likely to represent T-cells. The fact that CXCR3 and CCR5 are upregulated on T-cells also allows direct comparisons between α and β chemokine receptor signalling. These receptors recruit different G proteins and there is some evidence that signalling to non-receptor tyrosine kinases may also differ [96]. Most importantly, the signalling pathways for CC- and CXC-chemokine receptors can be compared and contrasted in these cells.

Activated Peripheral Blood Lymphocytes migrated towards 20 nM CXCL11 (Figure 4.2a) but unlike with THP-1 cells, pre-treatment of activated PBLs with 10 μ M gallein resulted in a significant reduction in migration vs. stimulated vehicle controls ($p \leq 0.01$, $n=3$). This was clear evidence that α chemokine receptors function differently from β chemokine receptors in chemotaxis and that G $\beta\gamma$ is required in some chemokine receptor signalling to chemotaxis. The effect of gallein pre-treatment on CXCL11 and CCL3 stimulated calcium release showed non-significant differences: 100 nM CXCL11 showed marked but non significant increase vs. the normalised control when treated with 10 μ M Gallein ($p > 0.05$, $n=3$). CCL3 was also capable of inducing calcium release in PBL's, suggesting that CCR5 or CCR1 was indeed expressed, but this calcium release was not affected by gallein treatment ($p > 0.05$, $n=3$).

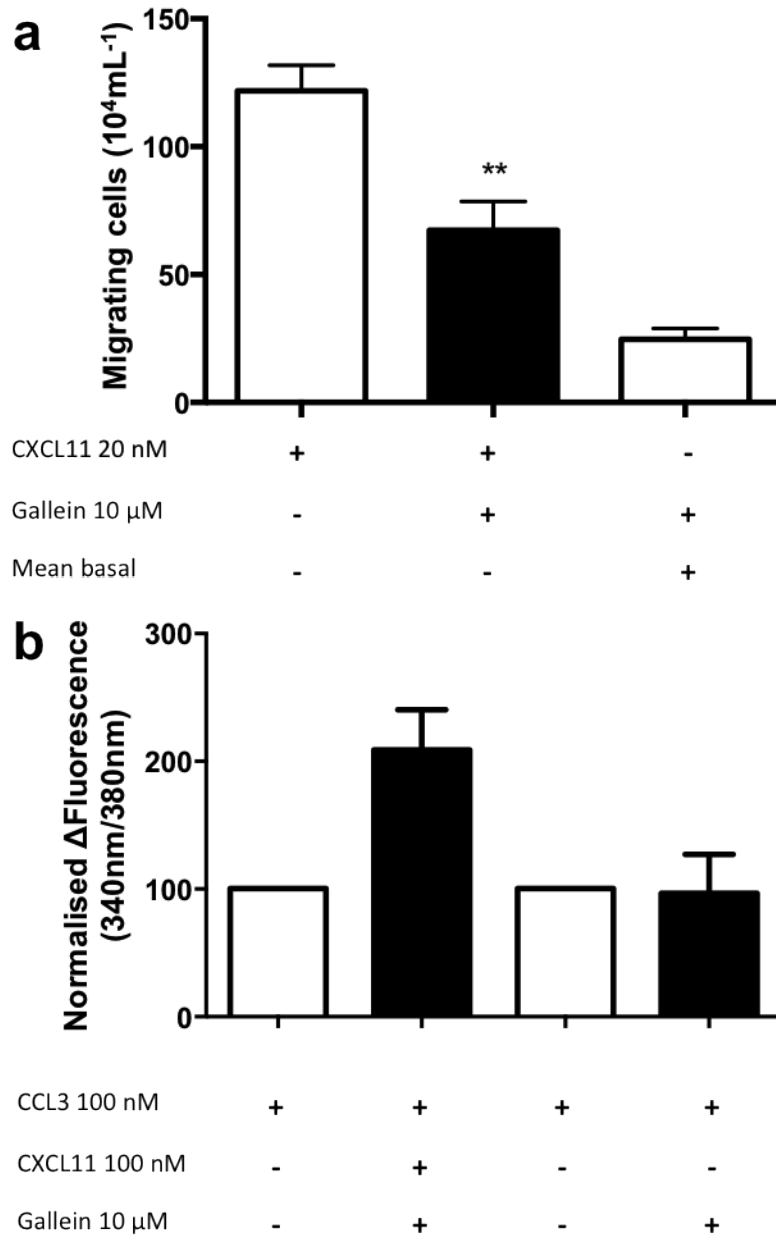


Figure 4.2: Gallein significantly reduces activated Peripheral Blood Lymphocytes chemotaxis towards CXCL11 but increases calcium release. (a) Activated Peripheral Blood Lymphocytes (PBLs) treated with 10 μ M Gallein show significantly reduced ability to migrate towards CXCL11 vs. vehicle control. (b) PBL calcium release stimulated by 100 nM CXCL11 show a trend towards increase by treatment with Gallein stimulation but 100 nM CCL3 is unaffected by Gallein. All results represent the mean \pm SEM of at least 3 donor's PBLs where n=1 is the mean of 3 independent experiments for 1 donor's PBLs. (One-way ANOVA, Bonferroni multiple comparison, ** = $p \leq 0.01$).

4.3.2. Investigation of arrestin recruitment in THP-1 cell chemotaxis

Non-visual arrestins are known to function either as mediators of GPCR desensitisation and internalisation or independently as scaffolds for signalling. The two functions of arrestin allow cellular responses to be tied directly to either arrestin dependent or 'classical' heterotrimeric G protein dependent signalling. Overexpression of arrestins should have opposing effects with each type of processes: enhancing arrestin dependent signalling and reducing 'classical' signalling by enhancing homologous GPCR desensitisation. To determine if CCR5 mediated calcium release was arrestin dependent cells were transfected (described in materials and methods) with either EGFP tagged arrestin 2 (Arr2) or arrestin 3 (Arr3) plasmid DNA. Control cells were transfected with pEGFP.C2 plasmids, which would express green fluorescent protein but have no signalling function.

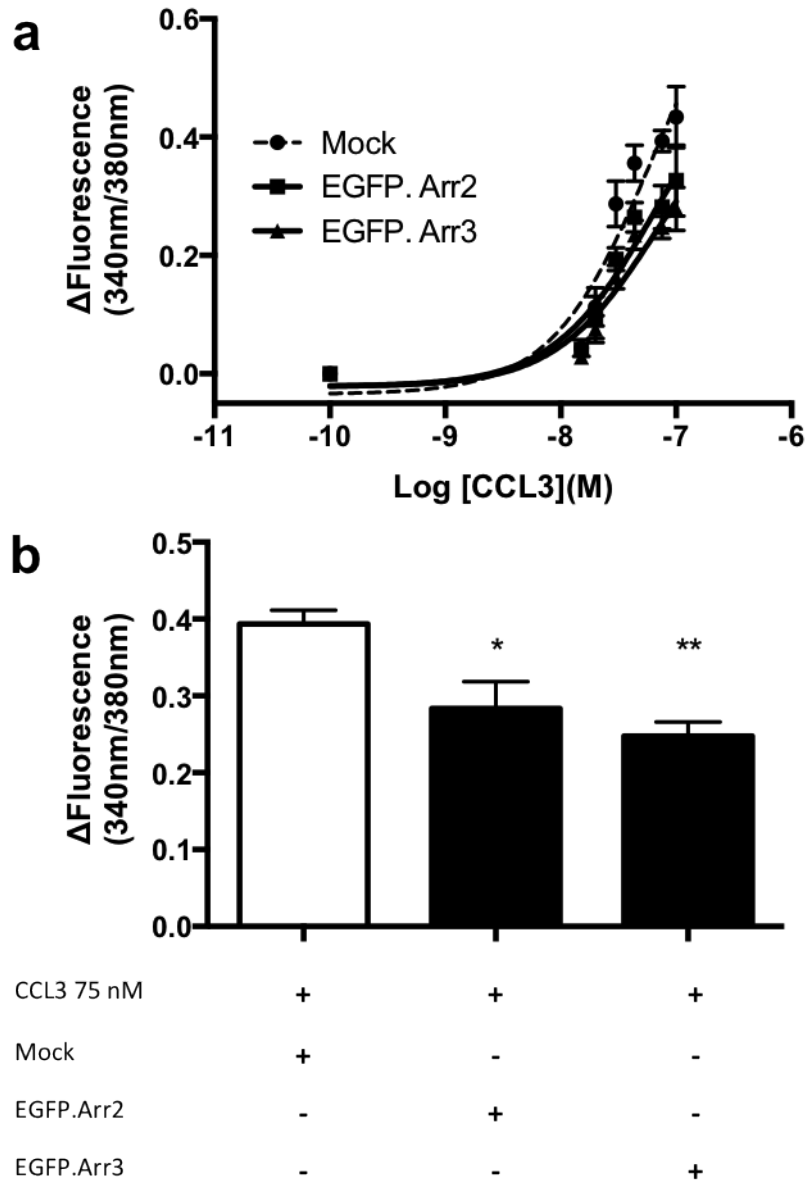


Figure 4.3: EGFP Arrestin 2 and 3 overexpression in HeLa.RC49 significantly reduces the ability of CCL3 to stimulate calcium release. (a) Concentration response curve for calcium mobilisation in HeLa.RC49 electroporation transfected with plasmid DNA coding for EGFP tagged arrestin 2 and 3 (EGFP.Arr2 & EGFP.Arr3) and non functional pEGFP.C2 as a mock control. The transient transfection of EGFP.Arr 2 & 3 resulted in a significant reduction in calcium release stimulated by 75 nm CCL3 ($p \leq 0.05$ vs. C2.EGFP transfected control) Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison* = $p \leq 0.05$, ** = $p \leq 0.01$ vs. control).

HeLa.RC49 transiently overexpressing arrestin 2 or 3 (Figure 4.3) showed no significant shift in CCL3 EC₅₀ for calcium release with values calculated as 61.7±4.8 nM for mock controls, 63.5±8.7 nM for arrestin 2 transfection and 65.5±5.3 nM for arrestin 3 transfection (assuming Hill coefficient of 1). Predicted efficacies for the three treatments were not significantly different (p.0.05) there was, however, a significant reduction in the level of calcium release for both arrestin isotypes vs. control THP-1 cells stimulated by 75 nM CCL3. Arrestin 2 overexpression reduced significantly calcium release by 28±10% (p≤0.05) and arrestin 3 overexpression significantly reduced calcium release by 37±7% (p≤0.001). These data indicate that CCR5 mediated calcium release in HeLa cells occurs via 'classical' G-protein signalling with arrestin acting as a desensitising protein, the difference in reduction of calcium release between the arrestin isotypes cannot be accounted for as differences in expression level of both arrestins were not quantified.

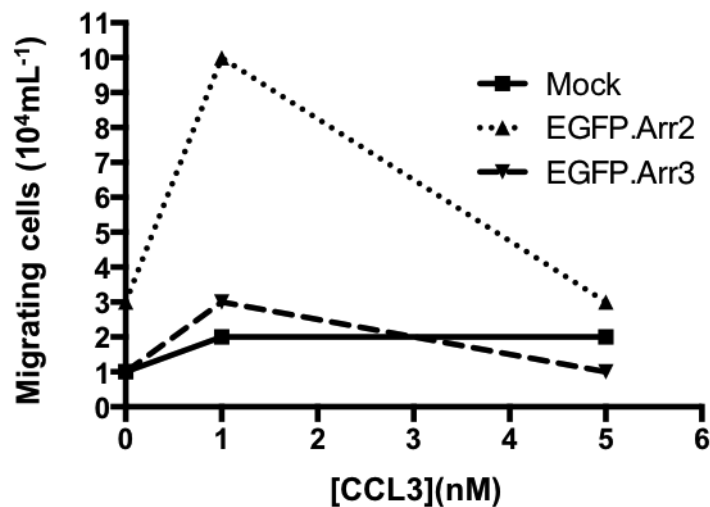


Figure 4.4: Electroporation transfection of THP-1 affects their ability to migrate. (a) Single experimental plot of THP-1 migration towards 0, 1 and 5 nM after transient transfection with EGFP.Arr2&3 and C2.EGFP control. Electroporation transfection resulted in a disruption in control migration in THP-1 with cells often not migrating at all. Plot represents typical migration. Note control stimulated migration 10 fold lower than expected.

Arrestin overexpression appeared to enhance CCR5 desensitisation, indicating mediation of calcium release occurred via 'classical' G-protein signalling, however, it is important to determine if chemotaxis occurs in an arrestin dependent or independent fashion. This is particularly important if signalling to chemotaxis can be shown to be divergent at the arrestin level then this might explain why calcium release and migration are independent in THP-1 [74]. THP-1 cells were electroporation transfected in the same way as for calcium release, however the process of transfection appeared to disrupt the ability of THP-1 cells to migrate. Figure 4.4 demonstrates representative results from transfected THP-1 cells in the transwell migration assay; control stimulated migration was reduced to levels 10 times lower than normal basal migration. Cells in some assays showed no migration at all, which demonstrated the unreliability of transfected THP-1 cells in this assay. Other methods of transfection, via chemical reagent, were available but the efficiency of these protocols had been shown to be low with THP-1 (data not shown), which supports the consensus idea that THP-1 are difficult to transfect effectively [228]. The inability to directly observe the effects of arrestin overexpression in THP-1 were disappointing but indirect methods of determining arrestin function were available with the use of inhibitors of GRK2 and PCK which act as 'recruitment kinases' for arrestins to GPCR C-termini.

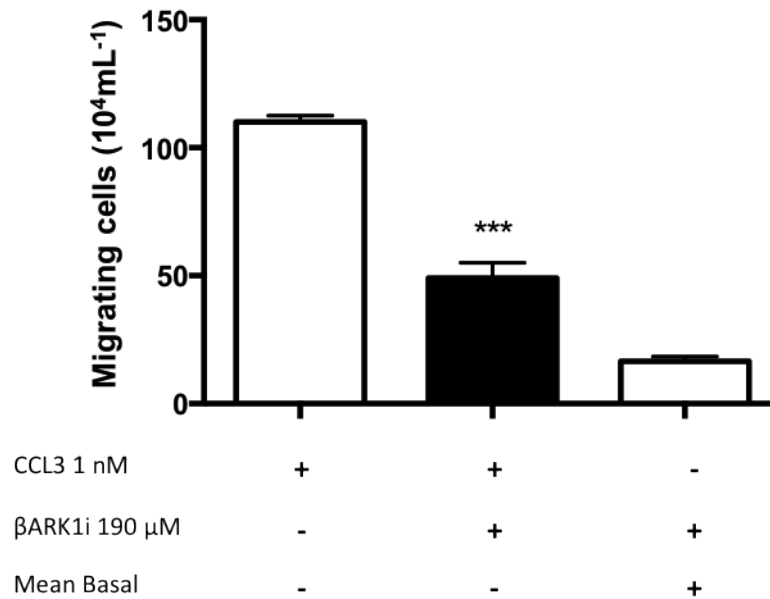


Figure 4.5: Inhibition of GRK2 with β Adrenergic Receptor Kinase inhibitor 1 significantly reduces chemotaxis in THP-1. Pre-treatment of THP-1 with 190 μM β Adrenergic Receptor Kinase inhibitor 1 (βARK1) significantly reduced migration toward 1 nM CCL3 vs. vehicle control. Results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * indicates $p \leq 0.001$ vs. control).**

βARK1 inhibitor is a rationally designed small molecule inhibitor of β-Adrenergic Receptor Kinase 1 (later termed GRK2) with an $IC_{50} \approx 125 \mu M$ [233]. βARK1 inhibitor binds to the GRK2 ATP binding site preventing ATP turnover and therefore GRK2 kinase function. Due to limitations on the solubility of the inhibitor and the requirement to keep DMSO vehicle levels as low as possible a treatment concentration of 190 μM at 1% DMSO vehicle was the maximum used. Pre-treatment of THP-1 cells for 0.5hr prior to transwell chemotaxis assay stimulated by 1 nM CCL3 (Figure 4.5). βARK1 inhibitor significantly reduced the ability of THP-1 cells to migrate by $66 \pm 7\%$ ($p \leq 0.001$, $n=6$), which is representative of the expected reduction based on the IC_{50} . Cytotoxic effects of βARK1 inhibitor were determined with the MTS assay (data not shown) and no significant reduction in cell viability was seen after 5hrs. These data indicate that migration is GRK2 dependent which in turn suggests that recruitment of arrestin via GRK2 phosphorylation is a prerequisite for CCR5 signalling to chemotaxis. There is some evidence that GRK2 can act as an

independent signalling molecule, which must also be considered. These data cannot explain whether the observed affect is due to the inhibition of arrestin dependent signalling or whether the arrestin dependent desensitisation and internalisation is the signal transduction dependent factor. Protein kinase C (PKC) also recruits arrestins to GPCRs but with different kinetics to GRK2 [38]. Pan isotype PKC inhibition does not inhibit THP-1 directly but depletes internal calcium [75]. The involvement of specific isotypes has not been well characterised particularly as there is some evidence that PKC ζ is involved with CCR5 specific heterologous desensitisation events [234].

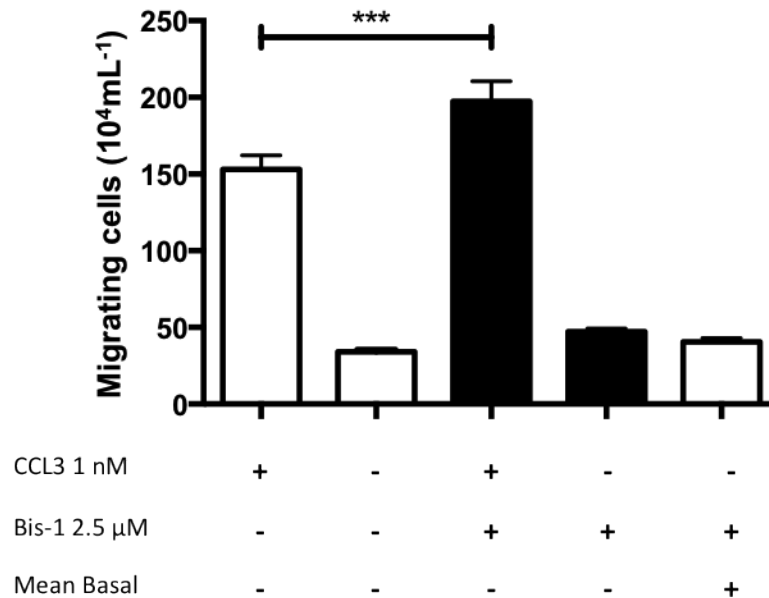


Figure 4.6: Pan-Isotype Protein Kinase C inhibitor bisindolylmaleimide significantly increases THP-1 migration towards CCL3. Pretreatment for 0.5hr with 2.5 μ M bisindolylmaleimide (Bis-1) significantly increases THP-1 chemotaxis towards 1 nM CCL3 vs. vehicle control. All results represent the mean \pm SEM of 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * indicates $p \leq 0.001$).**

Pre-treatment of THP-1 with the pan-isotype PKC inhibitor Bisindolylmaleimide (Bis-1) was used to determine the effect of general PKC function of CCR5 mediated signalling to chemotaxis (Figure 4.6). Treated THP-1 cells showed a significant increase in migration towards 1 nM CCL3

($p \leq 0.001$, $n=3$), which agreed with observations made by Cardaba *et al.* at higher inhibitor concentration [75]. This observation combined with those made using the GRK2 inhibitor suggest that arrestin recruitment via PKC and GRK2 leads to differential cellular responses with PKC mediated recruitment leading to desensitisation and GRK2 mediated signalling leading to arrestin dependent signal transduction to chemotaxis. This hypothesis is supported by investigation of the role of $G\beta\gamma$ signalling inhibition with gallein, which showed it was not required for CCR5 mediated chemotaxis, therefore activation of $G\beta\gamma$ by GRK2 was independent of migration[53].

At 2.5 μM Bis-1 will inhibit all conventional isotypes but not atypical protein kinase $c\zeta$. PKC ζ has been shown to be crucial for THP-1 migration mediated by cytokine receptors [235] so it was important to determine if inhibition of other PKC isotypes lead to altered PKC ζ function that was responsible for the observations seen with Bis-1 treatment. The cell membrane permeable oligomeric protein PKC ζ pseudo-substrate inhibitor (PKC ζ PSI)[236] was used to probe the involvement of this specific atypical PCK isotype on CCR5 signal transduction. Pre-treatment of THP-1 cells with 10 μM PCK ζ PSI for 0.5hr prior to intracellular calcium release assay (Figure 4.7) induced a significant reduction on calcium release stimulated by 75 nM CCL3 ($p \leq 0.01$, $n=3$) without affecting CCL3 EC_{50} values. Accordingly, predicted efficacy for CCL3 stimulated calcium release was significantly reduced (unpaired t-test, $p < 0.05$) with maximal ratiometric response over basal for control cells predicted as 0.573 ± 0.067 vs. 0.247 ± 0.035 for treated cells. As described in chapter 2 the possibility of these observations resulting from CCR1 specific signalling had to be considered so PCK ζ PSI was also used to treat HeLa.RC49.

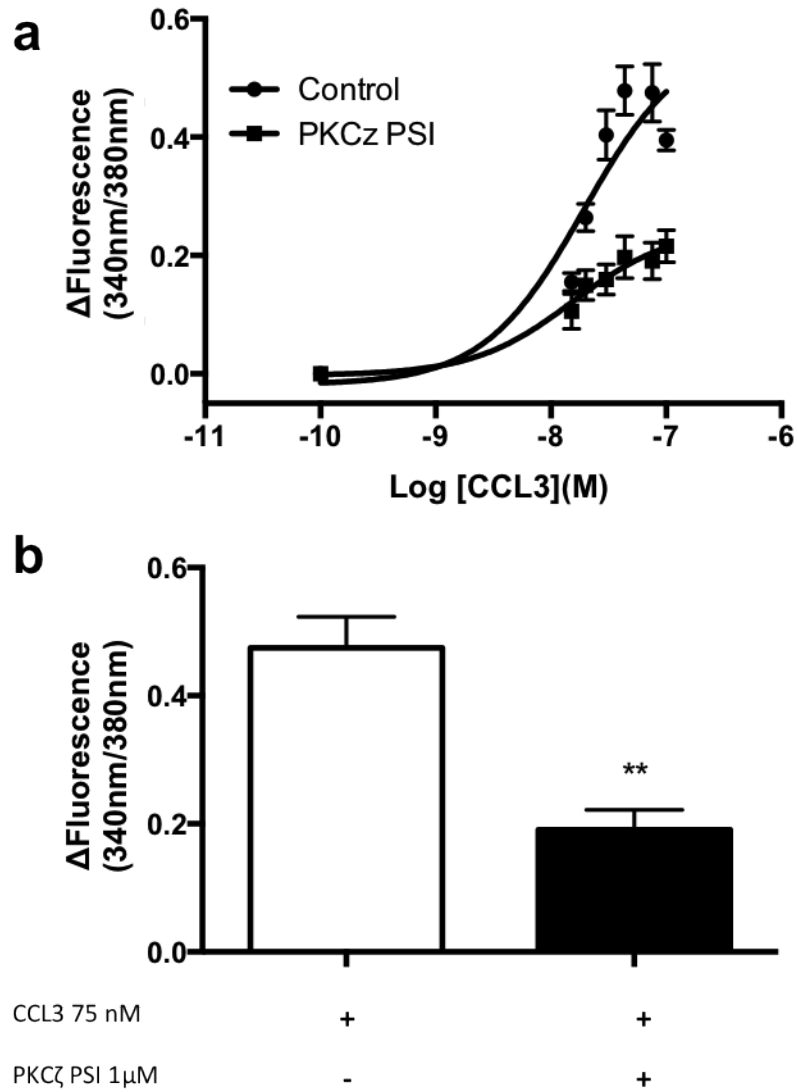


Figure 4.7: Inhibition of Protein Kinase C ζ with Protein Kinase C ζ pseudo substrate inhibitor significantly reduces calcium release in THP-1 cells. (a) Concentration response curve for calcium release in THP-1 pre-treated with 10 μ M oligomeric peptide Protein Kinase C ζ pseudo substrate inhibitor (PKC ζ PSI). PCK ζ PSI significantly reduces calcium release stimulated by 75 nm CCL3. Results represent the mean \pm SEM of at least 3 independent experiments (Unpaired t-test, ** indicates $p \leq 0.01$ vs. control).

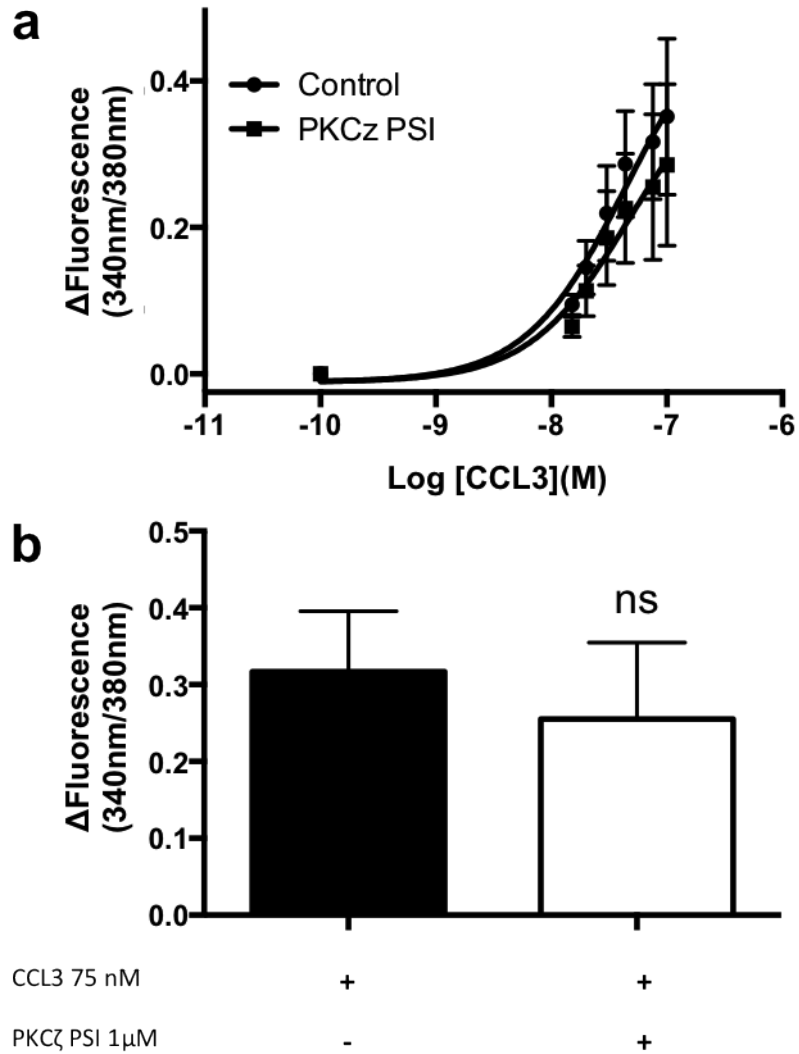


Figure 4.8: Inhibition of Protein Kinase C ζ with Protein Kinase C ζ pseudo substrate inhibitor does not affect calcium release in HeLa.RC49. (a) Concentration response curve for calcium release in HeLa.RC49 pretreated with 10 μ M oligopeptide Protein Kinase C ζ pseudo substrate inhibitor (PKC ζ PSI). PCK ζ PSI shows no significant effect on calcium release stimulated by 75 nm CCL3. Results represent the mean \pm SEM of at least 3 independent experiments (Unpaired t-test, ns= $p>0.05$).

Pre-treatment of HeLa.RC49 cells with 10 μ M PCK ζ PSI for 0.5hr had no significant effect on the EC₅₀ or the levels of calcium release observed (unpaired t-test $p>0.05$, $n\geq 3$) (Figure 4.8) and predicted efficacy was not significantly different between control and treated cells (unpaired t-test, $p>0.05$). This indicated that CCR5 mediated calcium release was either independent of PCK ζ mediated recruitment of arrestins or that PCK ζ was not involved in arrestin mediated desensitisation or signalling downstream of CCR5 in HeLa cells.

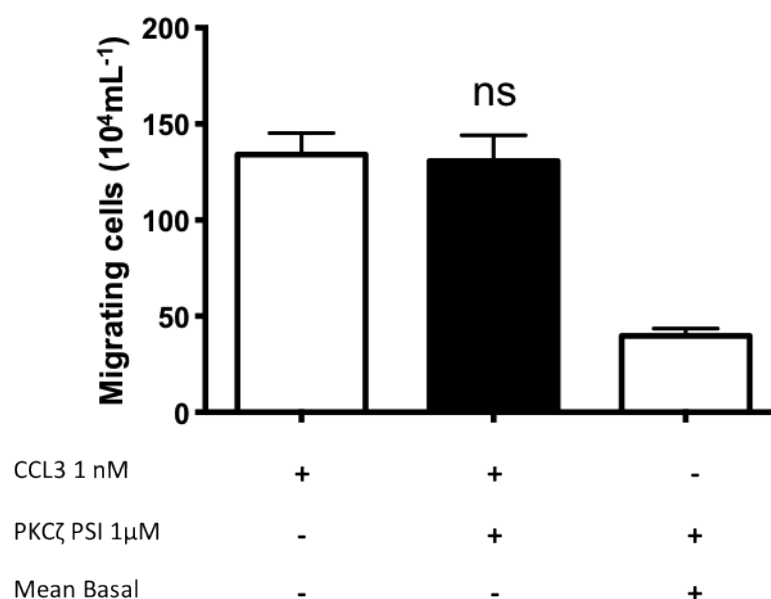


Figure 4.9: Inhibition of Protein Kinase C ζ with Protein Kinase C ζ pseudo substrate inhibitor does not affect CCL3 stimulated migration in THP-1. Pretreatment with 10 μ M oligopeptide Protein Kinase C ζ pseudo substrate inhibitor (PKC ζ PSI) does not significantly affect migration of THP-1 towards 1 nM CCL3. Results represent the mean \pm SEM of 6 independent experiments. (Unpaired t-test, ns= $p>0.05$).

The idea that PCK ζ is not involved in arrestin mediated desensitisation or signalling downstream of CCR5 is supported by the observed effect of 10 μ M PKC ζ PSI on THP-1 cell chemotaxis (Figure 4.9). Pre-treatment with PKC ζ PSI has no significant effect on THP-1 migration towards 1 nM CCL3 ($p>0.05$, $n\geq 3$) clearly showing that if PKC isotypes are involved in CCR5 signal transduction to migration PCK ζ is not one of them and provided further support for the idea that interplay between conventional PKC isotypes and GRK2 is responsible for the observed increase in migration THP-1 cells with bisindolylmaleimide inhibition.

4.3.3: Triangulating signalling to chemotaxis: MAPK, Rho and c-Src

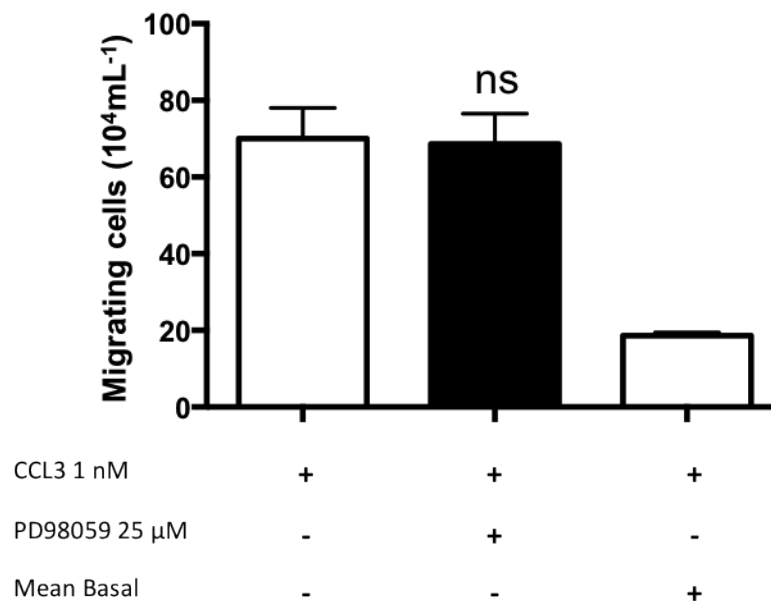


Figure 4.10: Extracellular Signal-Related Kinase (ERK) 1/2 inhibitor PD98059 does not affect THP-1 cell chemotaxis towards 1 nM CCL3. Incubation with 25 μ M PD98059 for 0.5hr did not significantly reduce the ability of THP-1 to migrate towards 1 nM CCL3. All results represent the mean \pm SEM of 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison, ns = $p > 0.05$ vs. vehicle).

ERK 1/2 and p38 Mitogen Activated Protein Kinases (MAPK) are associated with canonical $G\beta\gamma$ signalling to chemotaxis [57] and are also used more generally as markers of receptor mediated protein activation associated with $G\beta\gamma$ and arrestin dependent signalling [39, 43, 57]. It was important to determine if CCR5 signal transduction occurred through either of these proteins so that the role of phosphorylated MAP kinases in THP-1 chemotaxis would be better defined. PD98059 is a small molecule inhibitor of ERK1/2 MAPK phosphorylation and was used to pre-treat THP-1 cells at 25 μ M before chemotaxis assay. 0.5hr treatment with PD98059 had no significant effect on THP-1 migration towards CCL3 ($p \leq 0.05$, $n=3$) (Figure 4.10). The experimentation was repeated using the p38 MAPK inhibitor SB203580.

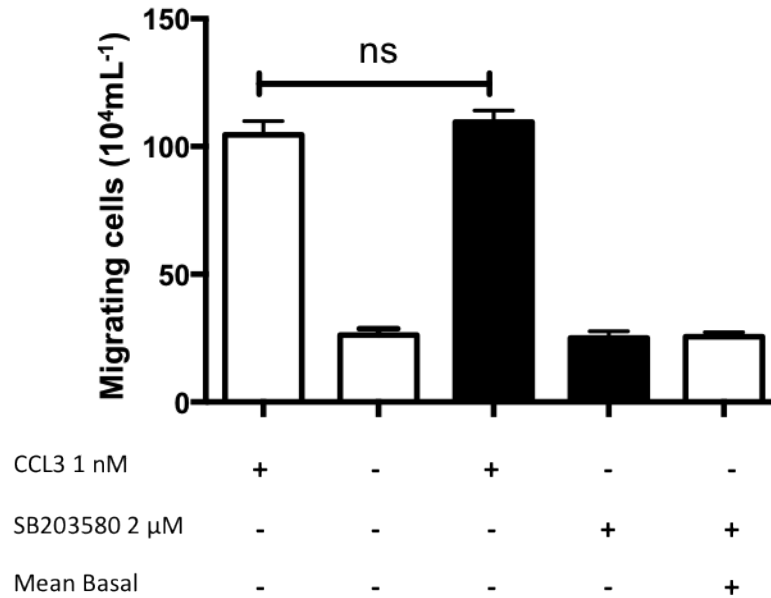


Figure 4.11: P38 Mitogen Activated Kinase inhibitor SB203580 does not affect THP-1 cell chemotaxis towards 1 nM CCL3. Incubation with 2 μM SB203580 for 0.5hr did not significantly reduce the ability of THP-1 to migrate towards 1 nM CCL3. All results represent the mean \pm SEM of 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, ns = $p > 0.05$).

SB203580 is a potent p38 MAPK inhibitor with an IC_{50} for p38 inhibition in THP-1 of cells \approx 100 nM [237]. THP-1 cells treated with 2.5 μM SB203580 prior to chemotaxis assay showed no significant alteration in migration compared with vehicle control cells (Figure 4.11) ($p > 0.05$, $n = 6$). Combined with the observations made with PD98059 inhibition, these data clearly indicate that chemotaxis signalling through CCR5 is independent of ERK1/2 and p38 MAPK which in turn supports the hypothesis that $\text{G}\beta\gamma$ signalling is also not required and chemotaxis and arrestin dependent signalling is responsible, in the first instance, for transduction of signals to cytoskeletal machinery and migration. These experiments suggest that CCR5 utilises atypical signal transduction to chemotaxis which is not described by the proposed transduction from activated G protein and is better explained by arrestin dependent signalling.

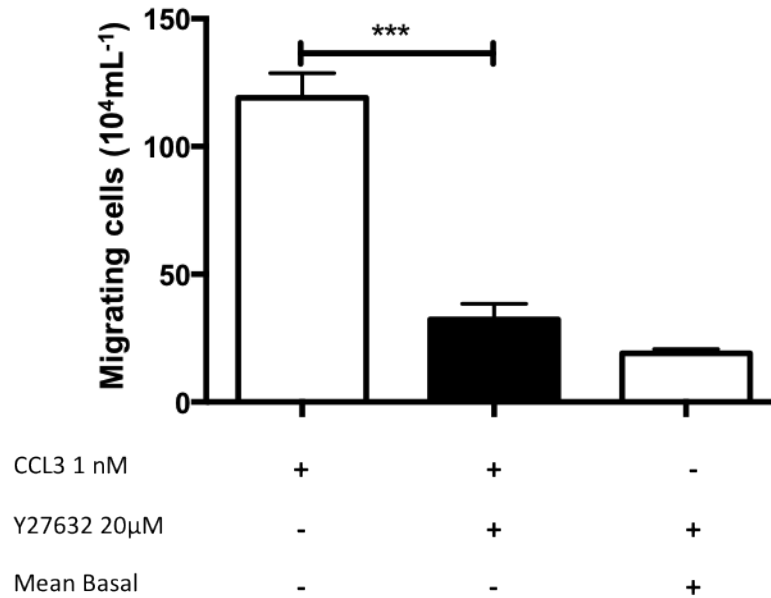


Figure 4.12: Rho Kinase (ROCK) inhibitor Y27632 significantly reduces THP-1 cell migration towards CCL3. Pre-treatment of THP-1 with 20 μM ROCK inhibitor Y27632 (Y276) significantly reduced their ability to undergo CCL3 stimulated chemotaxis vs. vehicle control. Results represent the mean \pm SEM of 6 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * indicates $p \leq 0.001$ vs. vehicle).**

The Rho family of small GTPases is generally considered crucial for chemotaxis with Rho responsible for actin dynamics and the disassembly of adhesions to allow progression of migration [192, 238, 239]. To determine if CCR5 mediated chemotaxis in THP-1 is dependent on Rho kinase, the small molecule inhibitor Y27632 was used to prevent Rho activation in THP-1 cells (Figure 4.12). Pre-treated THP-1 cells showed a significant reduction in migration towards 1 nM CCL3 ($p \leq 0.001$ $n=3$) that was not significantly higher than untreated basal migration ($p > 0.05$). These data indicated that CCR5 mediated migration shared some signalling in common with classical pathways.

Non-receptor tyrosine kinases such as Lyn and c-Src have been linked with CCR5 signalling [99] with Lyn in particular associated with the arrestin signalling scaffold identified by Cheung *et al.* [39] and signalling to Grb2 [100]. As ERK1/2 activation, does not seem to be involved in

migration (see Figure 4.10), the non-receptor tyrosine kinase bosutinib* was used to determine if non receptor tyrosine kinases of the c-Src/Lyn family are required for CCR5 mediated chemotaxis (*see Materials and methods for note on bosutinib isomerism issue).

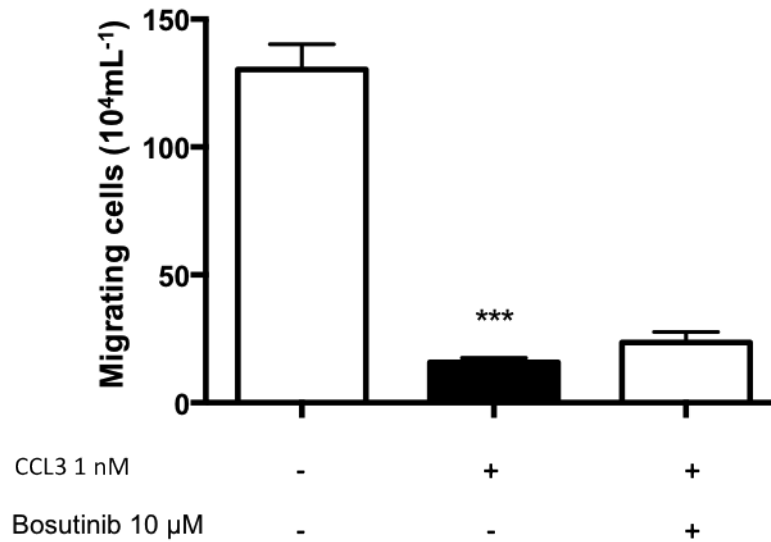


Figure 4.13: Non-receptor tyrosine kinase inhibitor bosutinib significantly reduces THP-1 cell chemotaxis. THP-1 pre-treated with the cellular sarcoma (c-Src) non-receptor tyrosine kinase inhibitor bosutinib do not migrate to a higher extent than the basal levels after activation with CCL3. Results represent the mean \pm SEM of 6 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * indicates $p \leq 0.001$ vs. vehicle).**

Bosutinib has been shown to be a potent inhibitor of Src family non receptor tyrosine kinases with an IC_{50} of around 100 nM and shows anti-proliferative effects in leukemia cell lines [79]. Pre-treatment of THP-1 with 10 μ M bosutinib significantly reduced the ability of THP-1 cells to migrate towards a 1 nM stimulus of CCL3 (Figure 4.13)($p \leq 0.001$, $n=6$). To determine if this observation could be attributed to disruption of CCR5 signal transduction a wound-healing assay using confluent monolayers of HeLa.RC49 was performed in the presence of bosutinib.

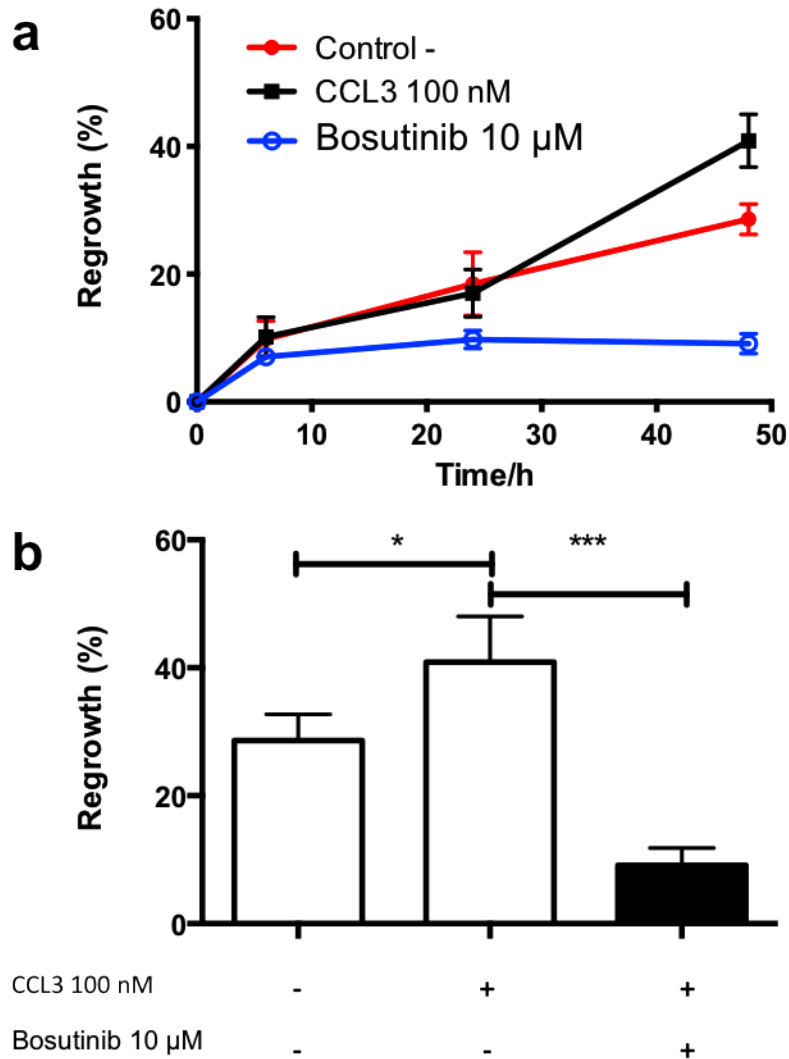


Figure 4.14: Bosutinib significantly reduces wound healing in HeLa.RC49 cells. (a) Graphical plot of HeLa.RC49 wound healing under basal and stimulated conditions and in the presence of 10 μM bosutinib. **(b)** Bosutinib treatment significantly reduced wound closing in a confluent monolayer of HeLa.RC49 to ≈ 10% after 48hr vs. CCL3 stimulated control. Results represent the mean ± SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison, * indicates $p \leq 0.05$ & *** ≤ 0.001 , $n=3$).

The wound-healing or scratch assay measures the ability of cells in a low serum environment to move into a freshly made scratch in a confluent monolayer. The maintenance of a low serum environment ensures that wound healing is mostly a result of cellular migration and not entirely due to proliferation. It should be noted that migration assays using 8 μm pore Corning HTS

transwell Boyden chambers were attempted with CHO.CCR5 and HeLa.RC49 cell but they proved to be unreliable (data not shown) and the scratch assay was adopted instead.

HeLa.RC49 cells exposed to bosutinib showed $\approx 10\%$ regrowth over 48hr, significantly less than the $\approx 40\%$ see with stimulated control cells ($p \leq 0.001$, $n=3$) (Figure 4.14). Bosutinib treated cell appeared normal throughout the duration of the assay with no significant alteration in cell morphology or signs of cytotoxicity such as detachment (not shown). These data supported the evidence provided by THP-1 cell treatment and suggested that bosutinib did affect CCR5 mediated migration by interfering with signal transduction and that c-Src family non-receptor tyrosine kinases were crucial to this signal transduction.

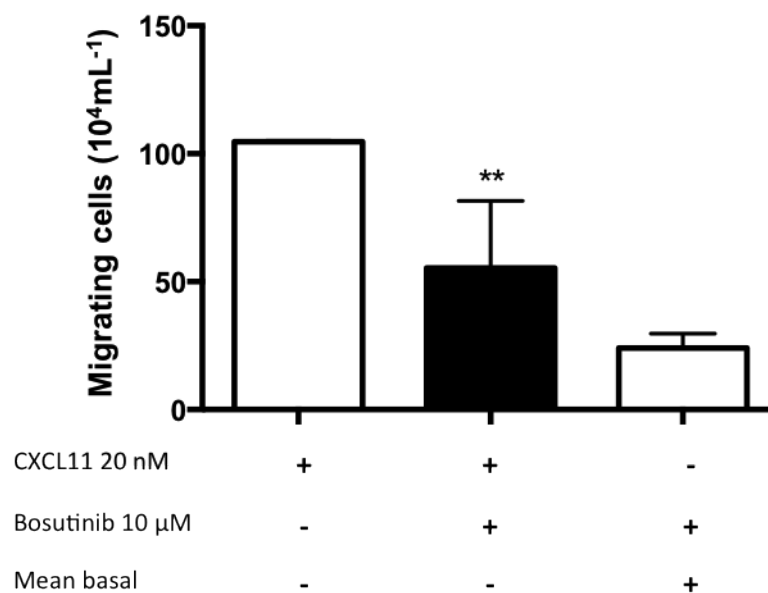


Figure 4.15: Bosutinib significantly reduces activated PBL chemotaxis. Activated PBLs pre-treated with bosutinib showed a significant reduction in chemotaxis towards 20 nM CXCL11. All results represent the mean \pm SEM of at least 3 donor's PBLs where $n=1$ is the mean of 3 independent experiments for 1 donor's PBLs. (One-way ANOVA, Bonferroni multiple comparison, ** indicates $p \leq 0.01$ vs. vehicle control).

Pre-treatment of PBLs with 10 μ M bosutinib resulted in a significant reduction in migration (Figure 4.15) ($p \leq 0.01$, $n=3$). The mean reduction in basal was not as low as that seen with THP-1, however, which may indicate that non-receptor tyrosine kinases are of less importance in CXCR3 mediated migration. A difference in donor PBL phenotype may also explain these observations.

4.4. Chapter discussion

There are three 'classical' routes through which CCR5 signal transduction could be communicated immediately downstream of the activated receptor: $G\alpha$, $G\beta\gamma$ or arrestin dependent signalling. Identifying which of these pathways is involved would be crucial to make sense of contradictory observations in the literature and will allow further investigation into the specifics of signal transduction to be targeted against specific interactions.

$G\beta\gamma$ subunits are widely associated with chemokine receptor signalling via activation of PI3K γ which signals to PKB and eventually to MAPK dependent signalling to cytoskeletal rearrangements and migration [57, 58]. The $G\beta\gamma$ inhibitor gallein has been shown to inhibit chemotaxis in other CCR systems [57] and represented an opportunity to disrupt signalling downstream of CCR5 without resorting to proteomic techniques. Gallein represents a useful investigative tool because the knock down of $G\beta$ and $G\gamma$ units via siRNA, which is the other alternative approach to this experiment, would be difficult due to the large number of isotypes and redundancy in signalling between isotypes [240]. Gallein treatment did not affect THP-1 cell chemotaxis which indicated the 'classical' signal transduction through $G\beta\gamma$ did not occur in this system. These data suggested that CCR5 mediated migration was effected by either $G\alpha_i$ or arrestin dependent signalling. Chemotaxis through CCR5 has been shown to be pertussis toxin (PTX) sensitive [74, 93] which would suggest that all signalling occurs via $G\alpha_i$, however, there are still questions regarding the nature of PTX inhibition, such as whether or not $G\beta\gamma$ and arrestin can function normally with covalently inactivated $G\alpha$ subunits interacting with the

receptor. With this in mind it was decided to investigate the possible role of arrestin dependent signalling to chemotaxis through CCR5 activation.

Non-visual arrestins 2 and 3 are ubiquitously expressed and highly researched mediators of GPCR signalling. In their canonical role 'arrest' GPCR activity by binding phosphorylated serine and threonine residues on the GPCR C-terminus [38] that leads to receptor desensitisation and internalisation [213, 241]. As described in the introduction, arrestins were shown to function as signalling scaffold with unique signalling capabilities; this arrestin dependent signalling was distinct from its role in internalisation [43] and is thought to be responsible for ligand-biased signalling observed through GPCRs [242, 243]. As discussed arrestin dependent signalling is important because 1) it may be arrestin isotype specific in some systems [243] and 2) it has been linked with signal transduction to chemotaxis [39]. There is evidence to support the idea that recruitment of arrestin isotypes is governed by GRK and PKC phosphorylation of the GPCR C-terminus and even that alterations to this phosphorylation can directly alternate signal transduction through specific arrestin isotypes [243].

Overexpression of arrestins 2 and 3 resulted in significant reductions in calcium release suggesting that activated CCR5 signalling to calcium release was dependent on classical G protein mediated signal transduction sensitive to arrestin mediated homologous desensitisation [43]. The effects of arrestin overexpression on migration could not be directly observed due to disruptive effects of the electroporation process. Indirect modulation of arrestin recruitment was attempted by treatment of THP-1 cells with the β ARK1 inhibitor which inhibits GRK2 phosphorylation of GPCR C-terminal domains. Although GRKs 2, 3, 5 and 6 have been shown to phosphorylate CCR5 C-terminal serines at supra-cytosolic concentrations [12] at normal cellular expression levels GRK2 is considered the primary mediator of arrestin recruitment [46]. β ARK1 significantly reduced THP-1 migration towards CCL3 suggesting that recruitment of arrestins enhanced signalling to chemotaxis therefore indicating that CCR5 mediated chemotaxis occurred via arrestin dependent signal transduction. These data, however, do not initially appear to support previous findings. Firstly GRK2 +/- murine t-cells, which express half cytosolic

concentrations of GRK2 showed significant increases in migration and ERK/PKB phosphorylation but ~50% reduction in CCR5 phosphorylation [232]. A possible explanation for these observations is that other GRK isoforms act redundantly or that alteration in the temporal kinetics of receptor phosphorylation/ arrestin recruitment are important to migration. Huttenrauch *et al.* demonstrated that CCR5 receptor mutants with C terminal serine/alanine mutations would recruit arrestins with any two of the four phosphorylation sites intact, but that receptor desensitisation required specific serine phosphorylation [43]. The reduction of GRK2 may therefore represent a reduction in desensitisation by arrestin binding whilst maintaining sufficient arrestin interaction to facilitate arrestin mediated signalling to chemotaxis. The second contradictory observation is that CCR5 C-terminal truncation mutants show significant increases in migration. Binding of arrestin solely to the ligand activated DRY motif may explain this observation and is supported by the fact that C-terminal induced desensitisation would be impossible allowing prolonged signalling. Arrestin mediated signalling scaffolds bound only via their secondary binding site to DRY motifs is an interesting idea and is supported the observation of Huttenrauch *et al.* that oligomeric protein sequences representing the DRY motif of CCR5 constitutively bind arrestin [43]. More relevant is to observation that CCR5 with a mutated DRY motif could not signal to chemotaxis [97]. The observed effects of C-terminal truncation also support the idea that the un-phosphorylated C-terminus acts as a negative regulator of signalling via CCR5 [43]. The observed effect of β ARK1 inhibitor treatment is explained in two ways, firstly the 190 μ M concentration is sufficient to inhibit over 50% of GRK2i therefore reducing any pro-migratory effects of limited GRK2 expression/inhibition as seen with GRK2^{+/-} mice. Secondly, as small molecule inhibition does not remove the GRK2 from the cytoplasm steric hindrance of localised but inactivated GRK might enhance inhibitory effects by preventing interaction between the receptor C-terminus and non-inhibited proteins. The effect of the pan-isotype PKC inhibitor bisindolylmaleimide may also support the role of GRK2 mediated arrestin recruitment in CCR5 mediated chemotaxis. Treatment of THP- cells with the inhibitor enhanced the migratory response towards CCL3, an observation which would be explained by a decrease in the rapid serine 337 phosphorylation known to occur via PKC [38] and a shift to the less rapid GRK2 mediated phosphorylation sites (Figure 4.16). If this were the

case, it may also suggest that GRK2 mediated phosphorylation of serine 337 may induce conformational changes that increase arrestin mediated signalling to migration. A further implication of these observations is that C-terminal phosphorylation at different loci can induce conformational changes in arrestin which can direct signalling to either desensitisation or arrestin dependent signalling. Another explanation for these observations however may be that the inhibition of the conventional protein kinase C with bisindolymaleimide enhances PKC ζ signalling. PKC ζ has been linked with migration in numerous cells types [236] and has been shown to specifically be required of THP-1 migration towards the cytokine CSF where it plays a role in facilitating actin polymerisation [235].

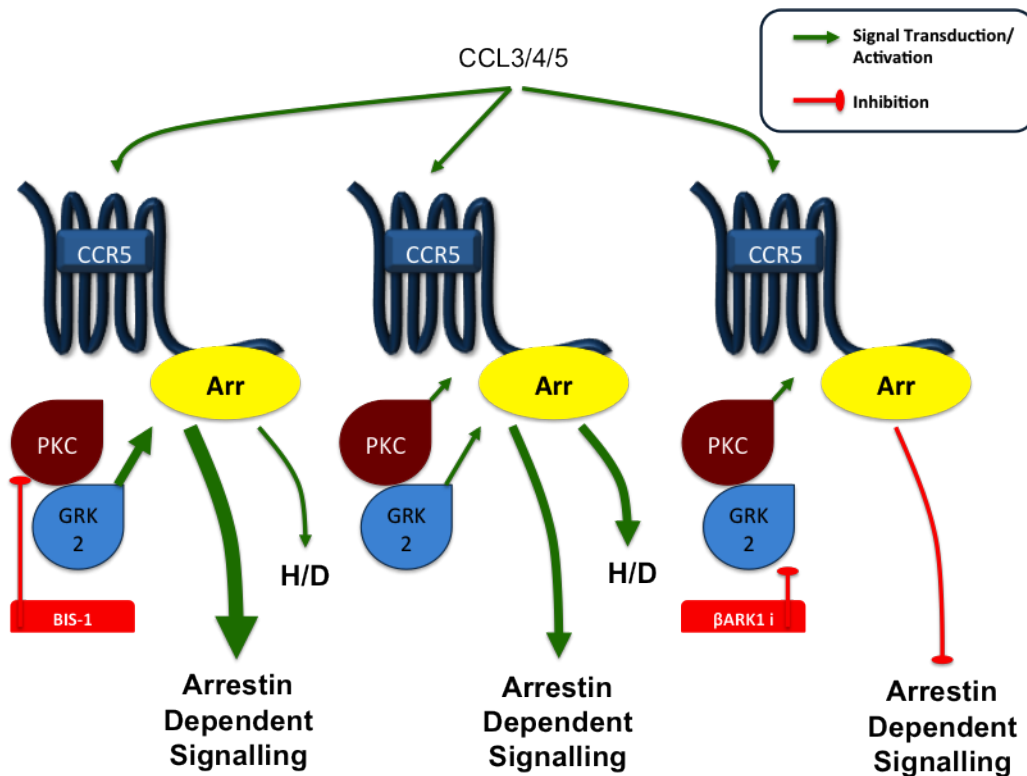


Figure 4.16: Possible explanations for the observed effects of GRK2 inhibitor β ARK1i and PKC inhibitor bisindolymaleimide. Usually CCR5 activation leads to arrestin mediated homologous desensitisation (H/D) or arrestin dependent signalling in a ratio determined by cytoplasmic protein complement. Treatment with bisindolymaleimide prevents rapid PKC phosphorylation of CCR5 and shifts signalling towards arrestin dependent signalling to chemotaxis. Treatment with β ARK1 inhibitor results in a decrease in all arrestin dependent processes.

Treatment of THP-1 cells with the oligomeric PKC ζ pseudosubstrate inhibitor significantly affects the efficacy of CCL3 stimulated calcium release but has no effect on migration. This effect on calcium reduction is not observed in similarly treated HeLa.RC49 cells suggesting that the observations seen in THP-1 cells are CCR1 specific or cell line specific. In either case, the data indicate that, unlike for CSF stimulated cytokine receptor mediated migration, PKC ζ is not required for CCR5 mediated migration in THP-1 cells. This also supports the idea that cytokine receptor transactivation, which has been shown to occur with numerous GPCRs [96] and CCR5 [85] specifically, is not involved in the chemotactic response. These observations also support the idea that bisindolylmaleimide treatment is reducing conventional PKC phosphorylation of the CCR5 C-terminus and altering arrestin recruitment kinetics. The observed effects of GRK2 and PKC inhibitors on THP-1 chemotaxis do not explain the role of arrestin mediated internalisation or the interaction with cytoskeletal mediators of the process such as clathrin and dynamin which may be instrumental in the migratory response [77].

ERK1/2 MAPK phosphorylation is often used as a marker for G $\beta\gamma$ and arrestin dependent pathway signalling and there are numerous studies suggesting a signal transduction axis for the ERK1/2 to chemotaxis via G $\beta\gamma$ or as part of arrestin signalling scaffolds [43]. However, particularly where arrestin dependent signalling is concerned, it is not clear whether ERK1/2 phosphorylation is a prerequisite of migration or a consequence of scaffold formation unrelated to migratory responses [39]. The potent ERK1/2 MAPK inhibitor PD98059 was used to investigate this and revealed that THP-1 migration via CCR5 activation is not sensitive to ERK 1/2 inhibition. Although this data may appear to suggest that either G $\beta\gamma$ and arrestin mediated signalling are not occurring, they can be interpreted as evidence to support arrestin scaffold signalling, as the non-phosphorylated ERK are still available to form signalling complexes with arrestin [39]. Conversely the 'linear' transduction from G $\beta\gamma$ –PI3K-PKB-ERK-migration associated with classical G protein signalling [57, 58] would be ERK activation dependent. Cheung *et al.* demonstrated that macrophage migration is dependent on both ERK and PI3K type 1A phosphorylation but it was not made clear if phosphorylation is required for scaffold formation. The potential diversity of these arrestin scaffolds was also not addressed; the calcium

dependent Pyk2 was shown to be important in the formation of the arrestin scaffold but in THP-1 cells, where calcium release and migration are known to be independent, alternative proteins may be recruited to arrestin scaffolds. For this reason it was important to determine the role of other MAPKs and reinforced the importance of understanding PI3K function downstream of activated chemokine receptors.

The p38 MAP kinase has been associated with α -chemokine receptor mediated arrestin dependent signalling [244] and with chemokine stimulated migration in vivo [245]. Therefore it was important to determine the effect of p38 inhibition in CCR5 mediated chemotaxis on THP-1. The potent small molecule inhibitor SB203580 was unable to affect THP-1 migration, suggesting the p38 protein played no role in the cytoplasmic (non-transcriptional) signalling to migration. The MAPK c-Jun N-terminal kinase (JNK) has been shown to associate with arrestin scaffolds [246] but it is associated with increases in transcription, which is unlikely to affect cytoplasmic signal transduction protein concentrations in the time frame of the chemotaxis assay. For this reason JNK inhibition was not investigated. The effects of ERK1/2 and p38 inhibition clearly show that although these proteins have been shown to be activated by arrestins and $G\beta\gamma$ their kinase function is not required for CCR5 mediated chemotaxis.

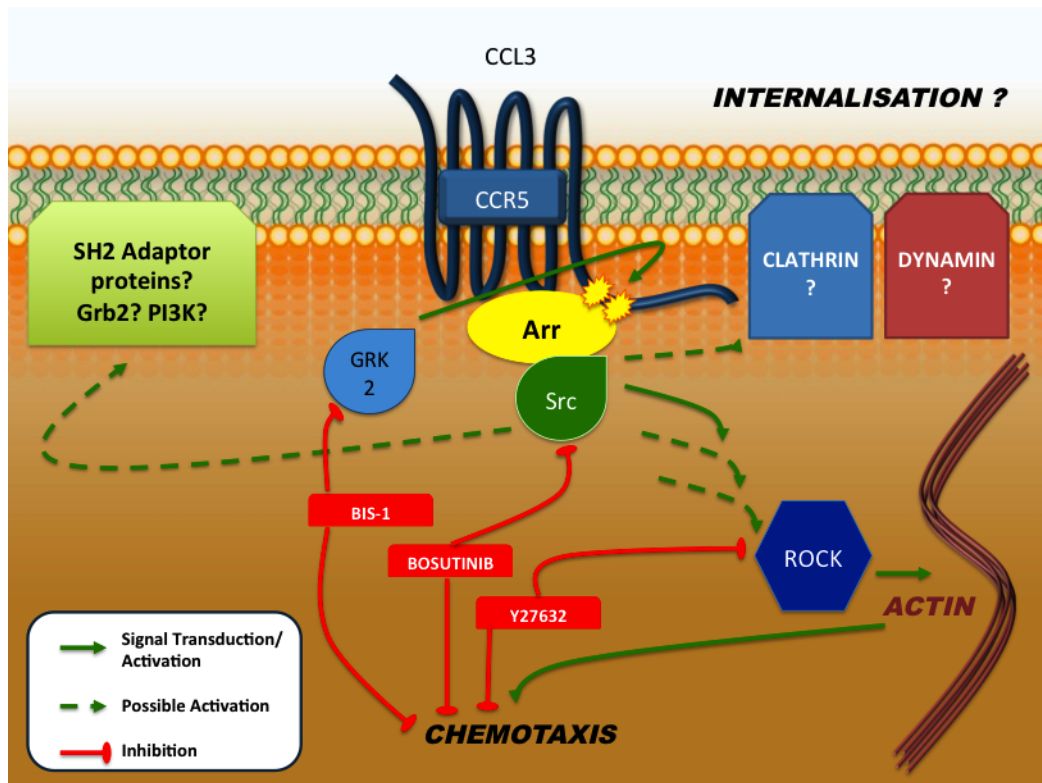


Figure 4.17: An Overview of chapter 4 results and possible lines of research. Arrestin dependent signalling directly or indirectly through Src family tyrosine kinases appears to represent an important axis in CCR5 mediated migration in THP-1 cells. (?) Represents interactions that should be investigated to clarify the nature of signal transduction between receptor activation and migration of cells.

The data outlined a picture of CCR5 mediated chemotaxis which seemed to vary from what might be expected based on what has been published about other GPCRs and chemokine receptors (Figure 4.17). It seemed unlikely that migration in THP-1 stimulated by CCL3 represented a unique signalling axis from start-to-finish, so it was therefore important to identify which signalling proteins further downstream are shared with canonical migratory signalling. Rho family proteins are known to be crucial for transduction into actin dynamics [247] and are involved with filopodia formation and focal adhesion disassembly [96, 192] and are linked to chemotaxis via numerous receptors types. Inhibition of Rho Kinase (ROCK) with the small molecule inhibitor Y27632 resulted in a significant reduction in migration demonstrating that downstream signalling from CCR5 converged at this point with other chemotactic

signalling. These data indicate that the novel CCR5 mediated signalling events to chemotaxis occur between the recruitment of arrestins and the activation of ROCK and that transactivation through receptor tyrosine kinases is not likely. The role of non-receptor tyrosine kinases in CCR5 mediated chemotaxis in THP-1 cells was investigated, as non-RTKs such as Lyn are important in migration [79]. Lyn forms part of the CCR5 associated arrestin scaffolded signalling complex identified by Cheung *et al.* [39], but have also been associated with trimeric G protein signalling [96]. The non-receptor tyrosine kinase inhibitor bosutinib has been shown to inhibit c-Src and Lyn with an IC_{50} around 500 nM [79]. Treatment of THP-cells with 10 μ M bosutinib significantly reduced migration, this indicates that non receptor tyrosine kinases are crucial for signalling to migration. These data do not explain how this may occur mechanistically: tyrosine phosphorylation is important in targeting proteins with SH2 binding sites such as PI3K regulatory domains, STATs and adaptor proteins such as Grb2. This suggested that a key process in CCL3 mediated migration might be the targeting of these adaptor protein complexes.

Bosutinib treatment was also effective at preventing HeLa.RC49 wound healing stimulated by CCL3, which suggested that non-receptor tyrosine kinase activity is important in CCR5 mediated migration in this system as well. Direct comparison between the two migration assays must be considered carefully for two reasons. Firstly there has been evidence to suggest that fibroblast and leukocytes migration requires different machinery [115, 248], with adherent cell migration being dependent on PI3K mediated cell polarisation, which is not important for monocyte migration [115]. Secondly, the extended time over which scratch assays occur allows increases in protein transcription stimulated by receptor activation to potentially alter the cytoplasmic signal transduction complement. For these reasons it was important to move the migration assay into cells which better represented THP-1 monocytes.

Activated peripheral blood leukocytes represent a population of primary human cells that are highly mobile and known to express CCR5 and CXC motif chemokine receptors [32]. Activated PLBs were used to determine if signalling through CXC motif chemokine receptors shared any similarities with CCR5 signalling to migration in small highly motile cells. Treatment of these

cells immediately revealed differences in cellular responses and gallein pre-treatment resulted in significant reductions in migration indicating 'classical' signalling to migration through Gβγ to PI3K, PKB and ERK. Calcium release in response to CXCL11 and CCL3 was also determined for activated PBLs. Both chemokines stimulated calcium release suggesting functional CC and CXC motif chemokines were present. Pre-treatment with gallein resulted in a non-significant increase in calcium release for both CCL3 and CXCL11 stimulated responses although a clear trend towards increase was observed with CXCL11 stimulated responses. The observed increase in calcium release has been noted in THP-1 signalling to calcium release in THP-1 cells so the observed response is not what would be expected (unpublished data, Kerr *et al.*) and likely to be due to differences in cell type what can be concluded is that the CCR5 and CXCR3 signalling functions differentially within a cell type which indicates that recruitment of signalling proteins to these receptors is not shared.

PBLs make a useful comparative system for CCL3 stimulated THP-1 cells without the disadvantages rationalising the differences between adherent vs. suspension cell chemotaxis [95, 115]. The use of PBLs also produces data with more clinical significance, as THP-1 cells, by their nature and despite validation as a model for disease, are abnormal.

The non-receptor tyrosine kinase inhibitor bosutinib, which significantly reduced migration in THP-1 cells to basal levels was used to determine if PBL migration was similarly affected. Significant reductions in migration towards CXCL3 were noted however the mean reduction was not as great as that seen in THP-1 cells (~60% reduction in PBLs vs. ~ 100% reduction in THP-1 cells). This observation may be explained by the fact that PBLs show large variability between donors in terms of the efficacy of bosutinib with one donor showing no reduction in migration. This variability can be attributed to genetic variability between donors but taken as a whole suggest that non receptor tyrosine kinases represent a common focus for signalling to migration downstream of both receptors like Rho Kinases are theorised to be.

Broadly, CCR5 signalling is known to occur via $G\alpha_i$, $G\beta\gamma$ or arrestin dependent pathways at the most 'upstream' level post receptor activation. Due to the potential misrepresentation of signalling which might occur with pertussis toxin treatment it was decided to focus on eliminating $G\beta\gamma$ and arrestin mediated pathways experimentally. THP-1 migration through CCR5 stimulation was shown to be Rho kinase dependent, which suggested activation of well documented chemotactic signalling at some level. Treatment with a $G\beta\gamma$ inhibitors and MAPK inhibitors suggested $G\beta\gamma$ signalling was not involved, which was interesting as this represented the 'classic' route to chemotaxis via chemokine receptors [43, 57]. The evidence strongly supported a role for arrestin dependent signalling however both at the recruitment and scaffold signalling level (Figure 4.17). These observations leave some unanswered questions however: the exact nature of arrestin in signal transduction is not clear, particularly with regard to any link to endocytosis or associated machinery such as clathrin and dynamin. This is because the observations made using GRK2 inhibitors could be also attributed to a failure of these processes affecting migration in some way. The role of non-receptor tyrosine kinases is also unclear as their function seems to be conserved in CXCR3 stimulated migration in activated PBLs. Non-receptor tyrosine kinase involvement with chemokine receptor mediated chemotaxis has been associated with $G\alpha$, $G\beta\gamma$ and transactivated cytokines receptors so more work is needed to unravel its true function in THP-1 cells. These questions will be addressed in chapter 6.

4.5. Chapter Conclusions

The final conclusions to be drawn from this chapter are:

- 1) The evidence supports that arrestin dependent signalling to chemotaxis through CCR5 in THP-1 cells represents the most likely route for signalling post receptor activation.
- 2) Downstream signalling through non-receptor tyrosine kinases and Rho kinase is also involved with signalling migration, which is also seen in CXCR3 mediated chemotaxis, and may therefore represent a common signal transduction pathway.
- 3) The role of internalisation and the mechanistics of non-receptor tyrosine kinases will be crucial in clarifying CCR5 mediated signal transduction.

CHAPTER 5 – JAK/STAT: Cytoplasmic mediators of chemokine receptor mediated chemotaxis?

5.1. Introduction

The JAK/STAT signalling axis consists of Janus Kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) which transduce signalling from activated membrane bound receptors to up regulate protein expression. JAK/STAT signalling is classically associated with activated cytokine receptors [119] and is well characterised as oncogenic in numerous cancers [126, 249]. Upon activation, cytokine receptors promote JAK autophosphorylation, that in time, leads to STAT phosphorylation and homodimerisation. STAT homodimers translocate to the nucleus to induce protein transcription for a range of proteins [126, 250]. Although JAK2 association with CXCR4 has been documented [251]; Mueller *et al.* demonstrated direct JAK2-CCR5 interaction [40], the nature of this association has not been well characterised. There is evidence to show CCR5 associated JAK2 activate STATs [122] and that this activation is G protein independent [40, 122]. If CCR5-activated JAK/STAT behaves solely as transcription factors or whether they can behave as cytoplasmic mediators of signal transduction to cellular responses is not known. JAK/STAT signalling has been associated with chemotaxis via a range of GPCRs [125, 252], and considering that signal transduction via G protein signalling to chemotaxis seem to be G-protein independent, the role of JAK/STAT in association with CCR5 makes an interesting target for investigation. JAK and STAT isotypes show preferential interactivity, with the canonical signalling of JAK1/STAT3 from type 1 cytokine receptors and JAK2/STAT5 through type 2 cytokines. These canonical pathways do not represent all possible JAK/STAT signalling with many combinations of JAK/STAT interaction appearing in the literature. The JAK2/STAT3 axis appears to be of clinical importance as it is associated with abnormal signalling in breast and other cancers [121, 126, 249] and STAT3 has associations with chemotaxis [252].

5.2. Chapter Aims

To determine if the JAK2 association, which has been shown to occur with CCR5, is involved in STAT3 activation or cytosolic signal transduction associated with CCR5 mediated chemotaxis.

A range of small molecule inhibitors of JAKs and STATs will be used to characterise calcium release and chemotaxis responses of THP-1 cells stimulated by CCL3.

5.3. Results

5.3.1: JAK2/STAT3 inhibitor cucurbitacin I completely inhibits CCR5 mediated chemotaxis by disrupting actin polymerisation

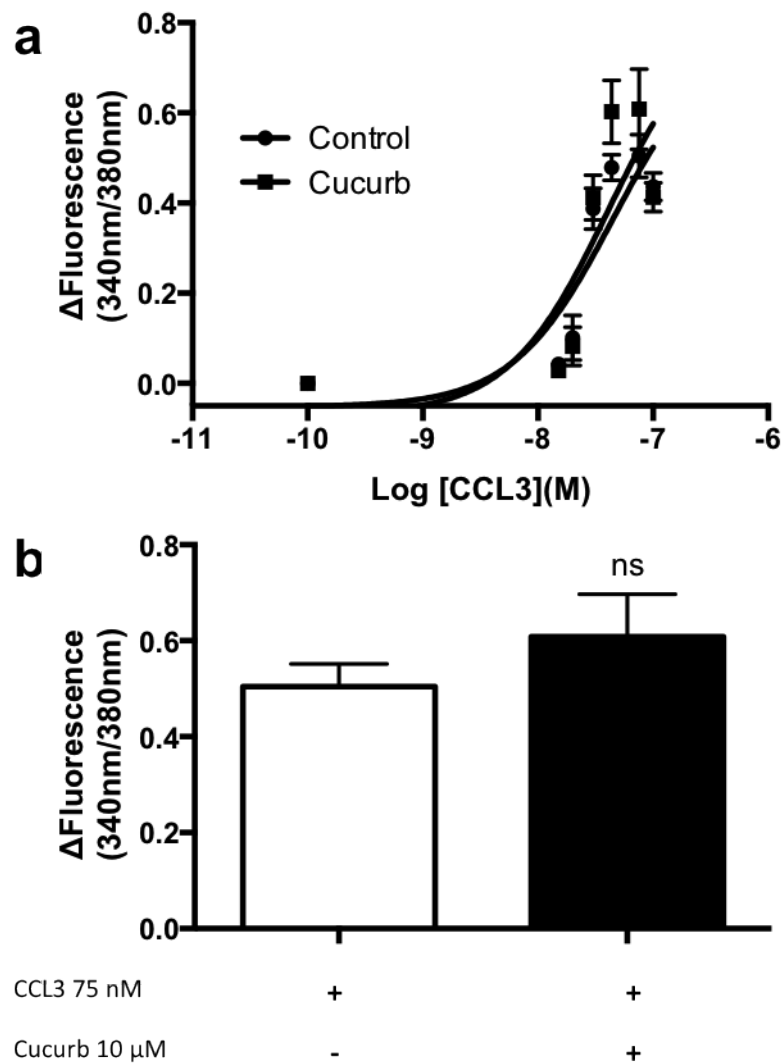


Figure 5.1: JAK2/STAT3 inhibitor cucurbitacin I does not significantly affect THP-1 cell calcium release stimulated by CCL3. (a) Concentration response curve for calcium release in THP-1 cells stimulated by CCL3 with and without treatment with cucurbitacin I (Cucurb). (b) Pre-treatment for 0.5hr with 10 μ M cucurbitacin I does not significantly affect intracellular calcium flux. Results represent the mean \pm SEM of at least 3 independent experiments (Unpaired t-test, ns = not significant vs. vehicle control).

Cucurbitacin I is one of a series of over 20 naturally occurring cucurbitacin homologues derived from the *Cucurbitaceae* (pumpkin) family of plants. Cucurbitacin I has been shown to inhibit phosphorylation of both JAK2 and STAT3 [126] and inhibits cellular functions dependent on JAK2/STAT3 signalling [253]. There is some evidence, however, that cucurbitacin affects actin fiber formation in some cell lines [254]. Pre-treatment of THP-1 cells with 10 μ M Cucurbitacin I for 0.5hr did not affect the concentration response of CCL3 stimulated calcium release (Figure 5.1a) (EC_{50} 45.5 \pm 9.6 nM control vs. 36.9 \pm 5.7 nM cucurbitacin I) and did not significantly reduce calcium release stimulated by 75 nm CCL3 (Figure J1b) (Students t-test $p > 0.05$ vs. vehicle control $n=3$).

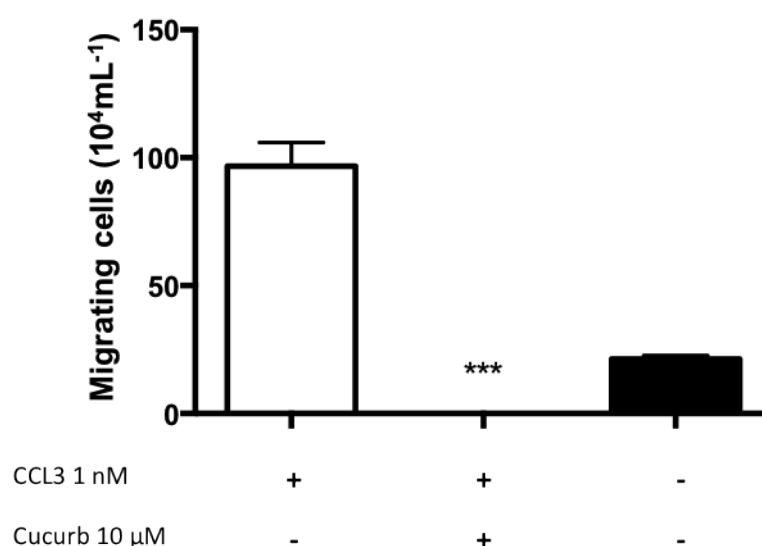


Figure 5.2: Cucurbitacin I completely inhibits CCL3 stimulated migration in THP-1 cells. Incubation with 10 μ M cucurbitacin I (Cucurb) for 0.5hr completely inhibited THP-1 cell migration 1 nM CCL3. All results represent the mean \pm SEM of 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. *** indicated $p \leq 0.001$, $n=3$).

Treatment with 10 μ M cucurbitacin had a marked effect on CCL3 mediated migration in THP-1, however, with migration in treated cells reduced to zero (Figure 5.2.). This marked reduction in chemotaxis could not be attributed to cytotoxic effect because calcium flux was unaffected

which suggested that the inhibitor was inducing a failure in cellular processes crucial to chemotaxis but not calcium release. Actin polymerisation was the obvious candidate: a functioning actin cytoskeleton is a prerequisite for all forms of cytoskeletal rearrangement and Knecht *et al.* had shown cucurbitacin I affected actin polymerisation [254].

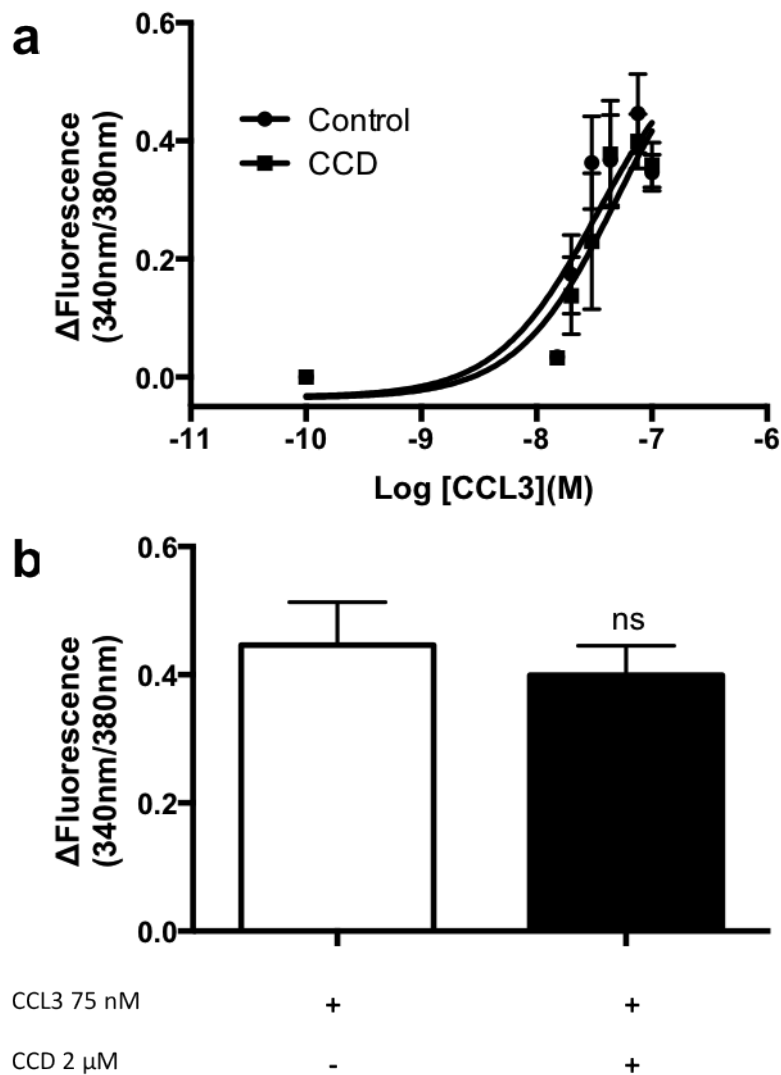


Figure 5.3: Actin polymerisation inhibitor cytochalasin D does not significantly affect THP-1 intracellular calcium release stimulated by CCL3. (a) Concentration response curve for calcium release in THP-1 cells stimulated by CCL3 with and without treatment with cytochalasin D (CCD). (b) Pre-treatment for 0.5hr with 2 µM cytochalasin D does not significantly affect intracellular calcium flux. Results represent the mean ± SEM of at least 3 independent experiments (Unpaired t-test. ns = not significant vs. vehicle p>0.05).

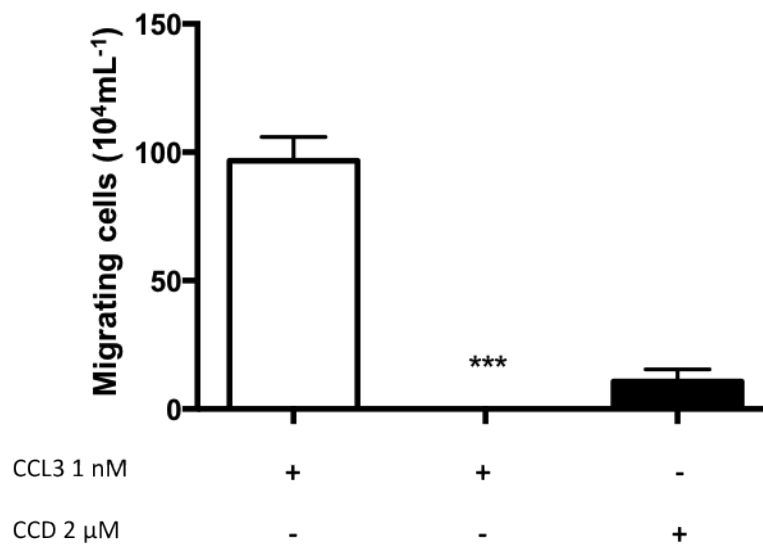


Figure 5.4: Cytochalasin D completely inhibits CCL3 stimulated migration in THP-1 cells. Incubation with 2 μ M cytochalasin D (CCD) for 0.5hr completely inhibited THP-1 to migration 1 nM CCL3. All results represent the mean \pm SEM of 6 independent experiments. (One-way ANOVA, Bonferroni multiple comparison. * = $p \leq 0.001$ vs. vehicle control).**

It was decided to use the actin polymerisation inhibitor cytochalasin D to draw comparisons with the effects of cucurbitacin I in THP-1 cells. Like cucurbitacin I, pre-treatment with the actin polymerisation inhibitor 2 μ M cytochalasin D had no significant effect on the EC_{50} (Figure 5.3a) or the level of intracellular calcium mobilisation stimulated by 75 nM CCL3. The effect of cytochalasin D pre-treatment on THP-1 chemotaxis was comparable to that seen with cucurbitacin I (figure 5.4). Migration towards 1 nM CCL3 was completely inhibited by the disruption of the actin cytoskeleton. Whilst this effect on migration is not surprising the fact that calcium release is completely independent of a functional actin cytoskeleton is remarkable and adds strong support to the conclusions of Cardaba *et al.* [75] that calcium flux and chemotaxis are independent phenomena in THP-1 cells.

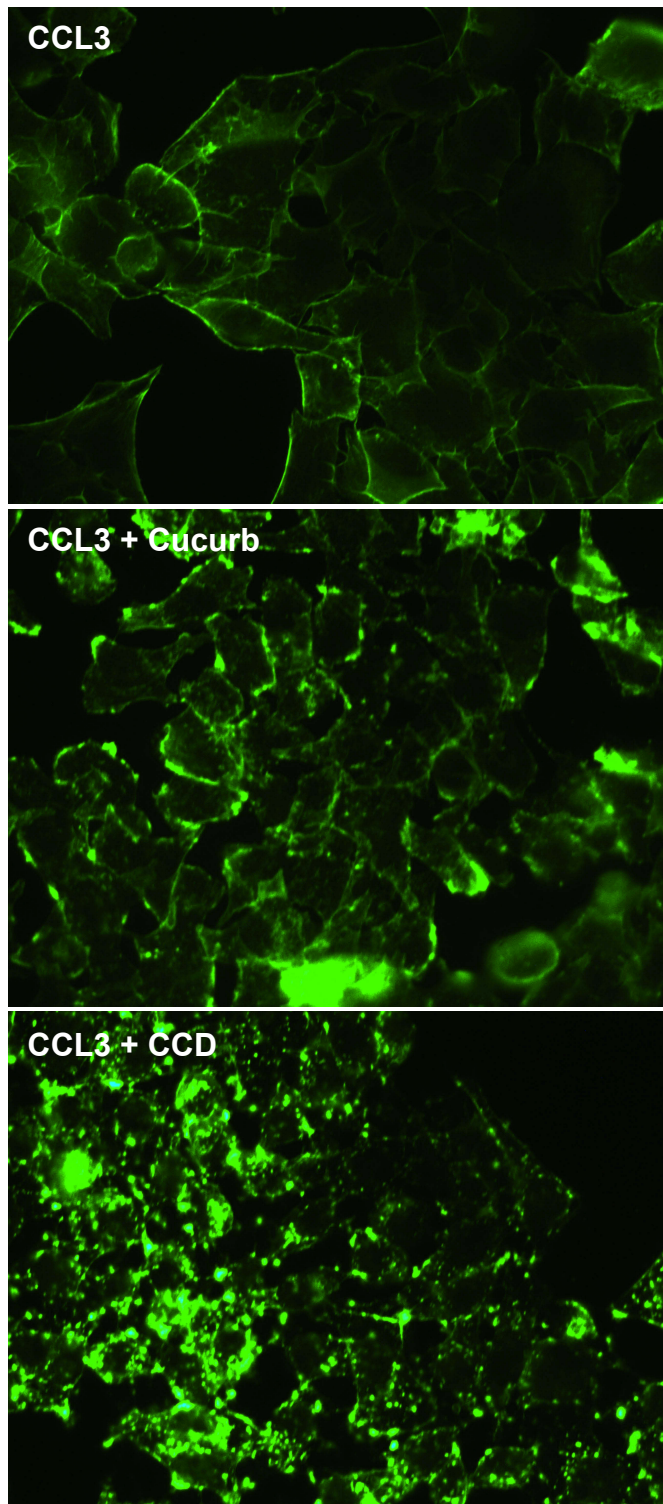


Figure 5.5: Cucurbitacin I disrupts actin polymerisation in HeLa.RC49 cells. Micrographs of HeLa.RC49 monolayers treated with 100 nM CCL3 and 10 μ M cucurbitacin I (Cucurb) and 2 μ M cytochalasin D (CCD). Cells were fixed and stained with Alexa-488 phalloidin actin stain (green). Images representative of population. Acquired with Leica imaging suite.

Fluorescence microscopy was used to determine if cucurbitacin I disrupts the cytoskeleton using Alexa-488 phalloidin stained HeLa.RC49 (Figure 5.5). These cells were grown as a monolayer on coverslips to high confluence (methods and materials) and were treated for 0.5hr with 10 μ M cucurbitacin I and then 100 nM CCL3 for 2hr prior to fixing and staining. Negative control cells were stimulated with 100 nM CCL3 for 2hr prior to fixing and staining and the positive control were pretreated with 2 μ M cytochalasin D for 0.5hr prior to 1hr CCL3 stimulation and staining. Although actin filaments were not widely evident in the negative control the distribution of actin was uniform. Cytochalasin D treated cells showed a marked disruption to actin localisation with no stress fibers evident and the uniformity of actin abrogated. Cucurbitacin I treated cells showed obvious disruption to the actin cytoskeleton although less pronounced than that seen with cytochalasin D treatment.

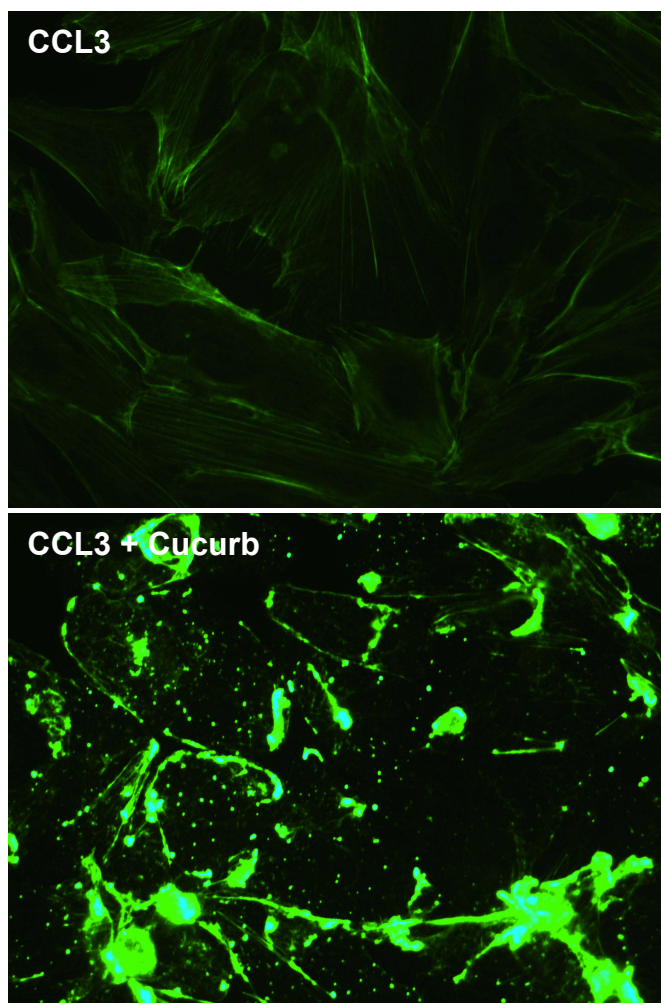


Figure 5.6: Cucurbitacin I disrupts actin polymerisation in CHO.CCR5 cells. Micrographs of CHO.CCR5 monolayers treated with 100 nM CCL3 and 10 μ M cucurbitacin I (Cucurb). Cells were fixed and stained with Alexa-488 phalloidin actin stain (green). Images representative of population. Acquired with Leica imaging suite.

The phalloidin actin stain was also carried out in CHO.CCR5 cells, which are larger and allow for easier identification of cellular structures. Alexa-488 tagged phalloidin stain in CCL3 activated CHO.CCR5 produced large numbers a clearly visible actin stress fibers (Figure 5.6). Treatment with 10 μ M cucurbitacin I completely disrupted the ability for actin fibers to form, clearly indicating that cucurbitacin I acts as an inhibitor or disruptor of actin polymerisation. Although it seems likely that disruption of actin was responsible for the blocking of chemotaxis in THP-1 cells, phalloidin staining of cucurbitacin I treated cells was inconclusive as the relatively small size of the cell made it difficult to distinguish differences between control and treated cell actin

structures (data not shown). It was decided that a pilot flow cytometry experiment would be run to determine if the technique could be used to differentiate between THP-1 cells with an intact and cytochalasin D disrupted cytoskeleton.

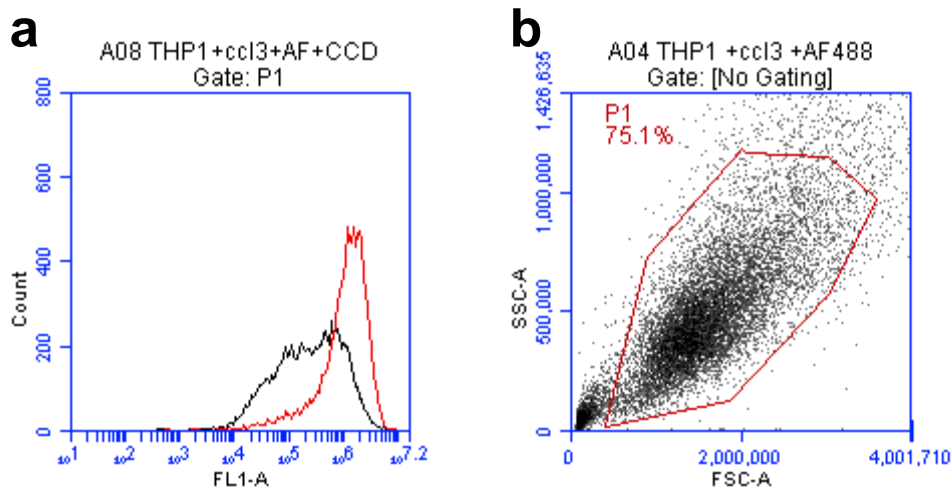


Figure 5.7: Cytochalasin D treatment produces a shift in phalloidin stained THP-1 fluorescence as determined by flow cytometry. (a) THP-1 treated with 2 μ M cytochalasin D show reduced fluorescence intensity (black trace) compared to untreated controls (red trace). (b) Front/Side scattering gating parameters.

THP-1 cells were activated with 100 nM CCL3 for 1hr before treatment with cytochalasin D (2 μ M for 0.5hr) and then stained with Alexa-488 phalloidin. Cytochalasin D treated cells showed a marked reduction in fluorescent intensity vs. control cells as determined by flow cytometry (Figure 5.7a) using the front/side scan gating shown (Figure 5.7b). The data indicated that flow cytometry could be used to analyse the effects of actin disruption in actin in THP-1 cells with sufficient difference between treated and untreated cells to allow intermediated effects to be distinguished. However, due to the relatively low resolution between control and cytochalasin D treated cells, and due to the fact that HeLa.RC49 and CHO.CCR5 imaging had revealed a notable disruption of the actin cytoskeleton with cucurbitacin treatment, it seemed reasonable to conclude that the same was happening with THP-1 cells.

5.3.2: JAK2 and STAT3 specific inhibitors have differential effects on CCL3 induced calcium release and migration in THP-1

A range of JAK2 and STAT3 specific inhibitors have been described [127, 128, 255] that represent a line of research that could allow the observations made with cucurbitacin I to be broken down and analysed in terms of specific proteins in the JAK/STAT axis. The inhibitors, known as JAK2 inhibitor 2, STAT3 inhibitor III and STAT3 inhibitor VIII target either JAK2 or STAT3 respectively, which would allow the roles of these proteins to be assessed individually. JAK2 inhibitor II or 1,2,3,4,5,6-Hexabromocyclohexane, targets JAK2 and prevents autophosphorylation with a maximal effect at 50 μM [127]. STAT3 inhibitor III is a small molecule tyrosine kinase inhibitor, which targets STAT3 and inhibits tumour cell proliferation with an IC_{50} of $\approx 5\mu\text{M}$ [255]. STAT3 inhibitor VIII is a porphyrin species which inhibits STAT3 SH2 binding but not other SH2 domains such as those on Grb2 at around $1\mu\text{M}$ and prevents STAT3 dimerisation with an IC_{50} of $10\mu\text{M}$ [128]. Therefore this inhibitor was used at $10\mu\text{M}$, in line with STAT3 inhibitor III, to simplify assay preparation.

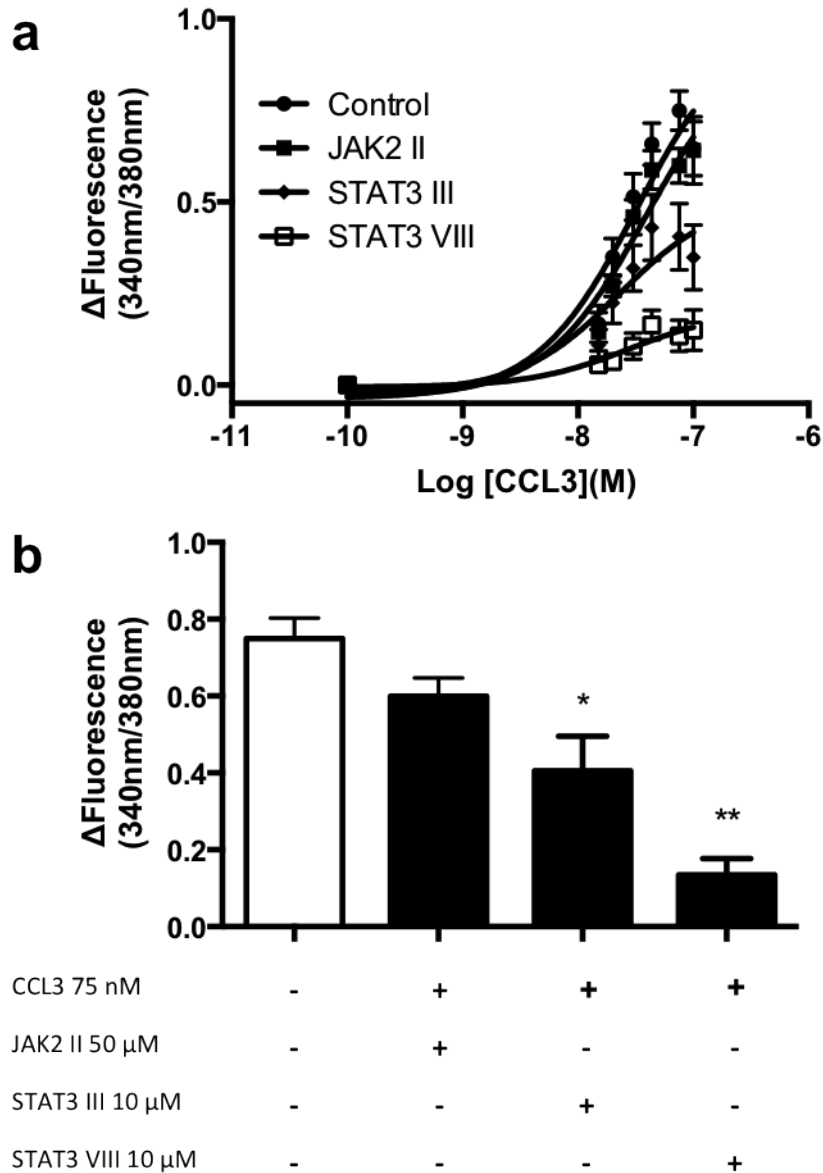
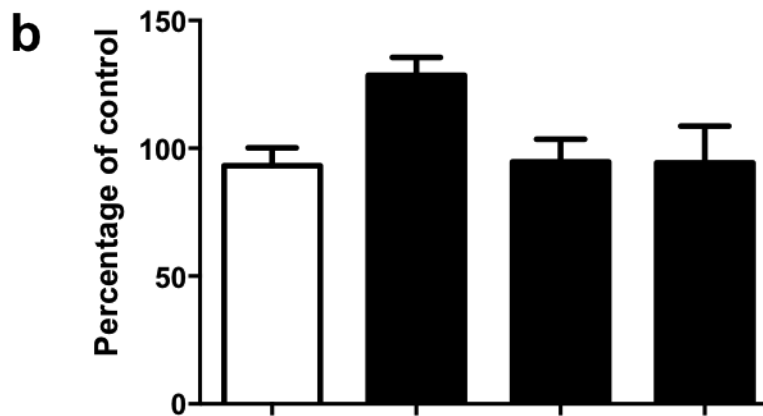
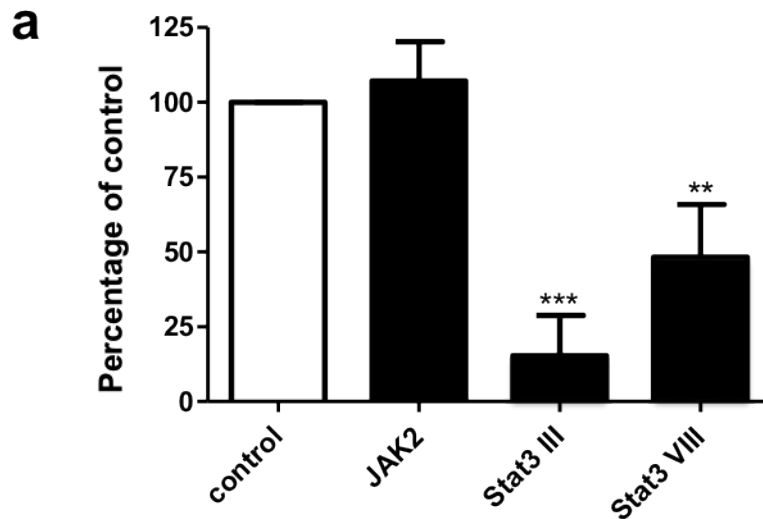


Figure 5.8: STAT3 inhibitors III & VIII but not JAK2 inhibitor II significantly reduce calcium release in THP-1 cells. (a) Concentration response curve for calcium release in THP-1 cells pre-treated with 50 μ M JAK2 inhibitor II (JAK2 II) or 10 μ M of either STAT3 inhibitors II or VII (STAT3 III & STAT3 VIII respectively). (b) STAT3 inhibitors significantly reduced calcium release in THP-1 cells stimulated by 75 nM CCL3. JAK2 II shows no significant effect. Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.05$ & ** $p \leq 0.01$ vs. vehicle control).

THP-1 cells were pre-treated for 0.5hr with the relevant concentrations of JAK2/STAT3 inhibitors before the release of intracellular calcium stimulated by increasing concentrations of CCL3 was determined. The concentration response curves for calcium release (Figure 5.8a) indicated the EC_{50} for all treatments was comparable to the vehicle control ($EC_{50} = 29.8 \pm 2.0$ nM (control) vs. 35.9 ± 6.2 nM (JAK2 II) vs. 20.9 ± 2.6 nM (STAT 3 III) vs. 35.2 ± 8.9 nM (STAT3 VIII)), however the level on calcium release was significantly lower at 75 nM CCL3 stimulation for cells treated with STAT3 inhibitors. STAT3 inhibitor III reduced calcium release induced by 75nM CCL3 in THP-1 cells by $46\% \pm 14$ vs. vehicle control ($p \leq 0.05$, $n=3$) and STAT3 VIII showed reduced calcium flux by $82\% \pm 9$ vs. vehicle ($p \leq 0.01$, $n=3$). This was supported by the calculated efficacy, that was significantly lower for STAT3 III and VIII compared to the vehicle bases on the sigmoidal concentration response fitted with an assumed Hill coefficient of 1. JAK2 inhibitor 2 showed no significant reduction in the level of calcium mobilisation stimulated ($p > 0.05$, $n=3$). These data are surprising considering that cucurbitacin I did not affect calcium release. If JAK2 inhibition had resulted in inhibition of calcium release and STAT3 had not then this would at least be consistent with a canonical JAK2/STAT5 dependent signalling axis but as JAK2 inhibition should result in indirect reduction of STAT3 phosphorylation these observations were puzzling. One explanation maybe a redundancy in the signalling axis with JAK1 acting in place of JAK2, however more investigation would be required to add support to this hypothesis. To determine if the inhibitors were reducing STAT3 phosphorylation in the model system, a STAT3 tyrosine 705 (Tyr705) phosphorylation ELISA (described in Materials and Methods) was employed to determine if phosphorylation of STAT3 was reduced in the presence of the inhibitors. STAT3 Tyr705 is required for STAT3 homodimer translocation to the nucleus and is used widely as a marker for STAT3 activation [256]. IL-6 was elected as a stimulator in this assay as it is well characterised as an activator of STAT3 via JAK2 and JAK3 [256] and would reduce the need to develop methodology to optimised stimulatory parameters of JAK2 via CCR5 activation.



IL-6 40 nM	-	+	-	-
STAT3 III	-	-	+	-
STAT3 VIII	-	-	-	+

Figure 5.9: STAT3 inhibitors III and VIII significantly reduce IL-6 stimulated STAT3 phosphorylation in THP-1 cells. (a) Significant reduction STAT3 Tyr705 phosphorylation as determined in the InstantOne™ ELISA. (b) STAT3 Tyr705 phosphorylation in HeLa.RC49 stimulated by 40 nM IL-6 is reduced to non-stimulated levels in the presence of Stat3 inhibitors III and VIII. Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. ** indicates $p \leq 0.01$ & * $p \leq 0.001$ vs. IL-6 stimulated control).**

STAT3 Tyr705 phosphorylation was stimulated by 40 nM IL-6 in THP-1 cells above the non-stimulated control and both STAT3 inhibitors were able to significantly reduce phosphorylation (STAT3 III $p \leq 0.001$, $n=3$. STAT3 VIII $p \leq 0.01$, $n=3$) however JAK2 inhibitor 2 had no significant effect on STAT3 phosphorylation (Figure 5.9a). The STAT3 inhibitors were also tested in HeLa.RC49 to determine if the inhibitors were effective between cell types and again showed the ability to prevent STAT3 Tyr705 phosphorylation vs. IL-6 stimulated controls although, due to the relatively low level of activation in HeLa.RC49 cells this relationship was observed as a non-significant trend ($p > 0.05$, $n=3$). Despite the lack of significance the data clearly confirm the result seen in THP-1 and indicate the effects of STAT3 inhibitors are not cell-type specific.

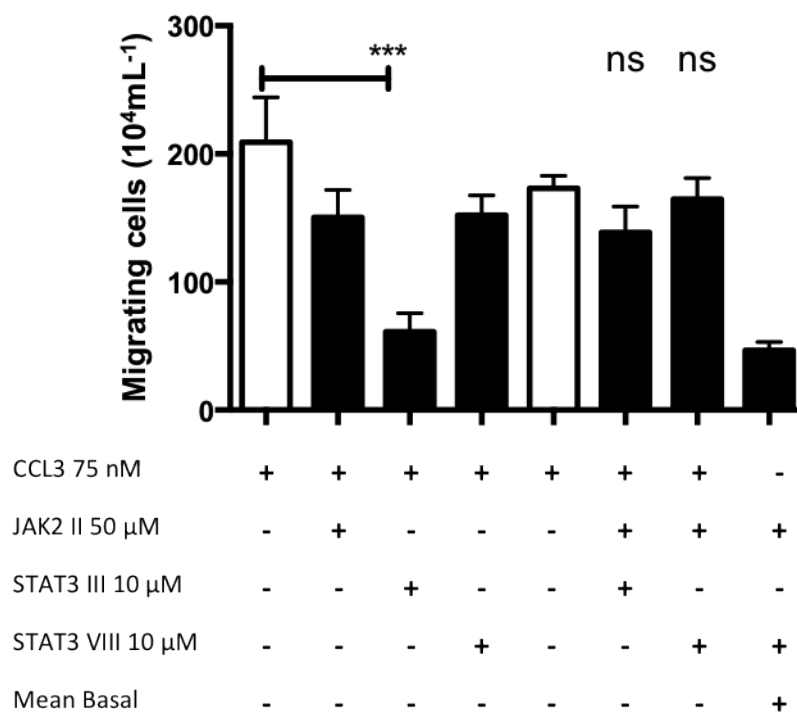


Figure 5.10: STAT 3 inhibitor III significantly reduces THP-1 chemotaxis. THP-1 pre-treated with STAT3 inhibitor III (STAT3 III) demonstrates a significant reduction in migration. THP-1 cells treated with JAK2 inhibitor II (JAK2 II), STAT3 inhibitor VIII (STAT3 VIII) and combinations of JAK2 + STAT3 III and JAK2 II + STAT3 VIII showed no significant reduction in migration. Results represent the mean \pm SEM of at least 3 independent experiments (***) indicates $p \leq 0.001$ vs. vehicle control, ns = not significant).

In order to determine if either JAK2 or STAT3 could be attributed to the effects seen using the combined JAK2+STAT3 inhibitor cucurbitacin I in chemotaxis, the JAK2 and STAT3 inhibitors were used to pre-treat THP-1 cells prior to a chemotaxis assay towards 1 nM CCL3. The inhibitors were also used in conjunction with one another (JAK2+STAT3 III and JAK2 + STAT3 VIII) to determine if the effects of cucurbitacin I could be replicated. Interestingly, only STAT3 inhibitor III treatment had a significant effect on migration ($p \leq 0.001$, $n=3$), but this effect was completely abrogated ($p > 0.05$, $n=3$ vs. vehicle control) when combined with JAK2 inhibitor II inhibition (Figure 5.10). The reason for this observation is not clear as a combined JAK2/STAT3 inhibition would be expected to reduce STAT3 phosphorylation but may be explained if JAK2 inhibitor 2 interacted with or allosterically out-competed binding of STAT3 inhibitor 3. Statistical comparison of the two treatments reveals that they are not significantly different from one another ($p > 0.05$), suggesting an aberrant result. This comparison is not ideal due to the different vehicle levels, but offers an explanation for the observation, which agrees with the other data. The other point to consider is that despite being shown to inhibit STAT3 Tyr705 phosphorylation, STAT3 VIII had no significant effect on migration, which suggests that either 1) the differences in STAT3 binding between the two compounds results in alterations in their functionality with other proteins or 2) STAT3 III has off-target or cytotoxic effects. Taken as a whole the data from the chemotaxis assays shows that JAK2 is not involved in cytoplasmic signal transduction to chemotaxis and that combined inhibition of JAK2+STAT3 does not replicate the effect on migration seen with cucurbitacin I. The function of STAT3 was less clear and warranted further investigation; the taught Master's degree student S. Khabbazi undertook the research into this question with supervision from the author. The resulting findings were published by Khabbazi et al. [47] and demonstrated that the effects of STAT3 III are due to cytotoxicity.

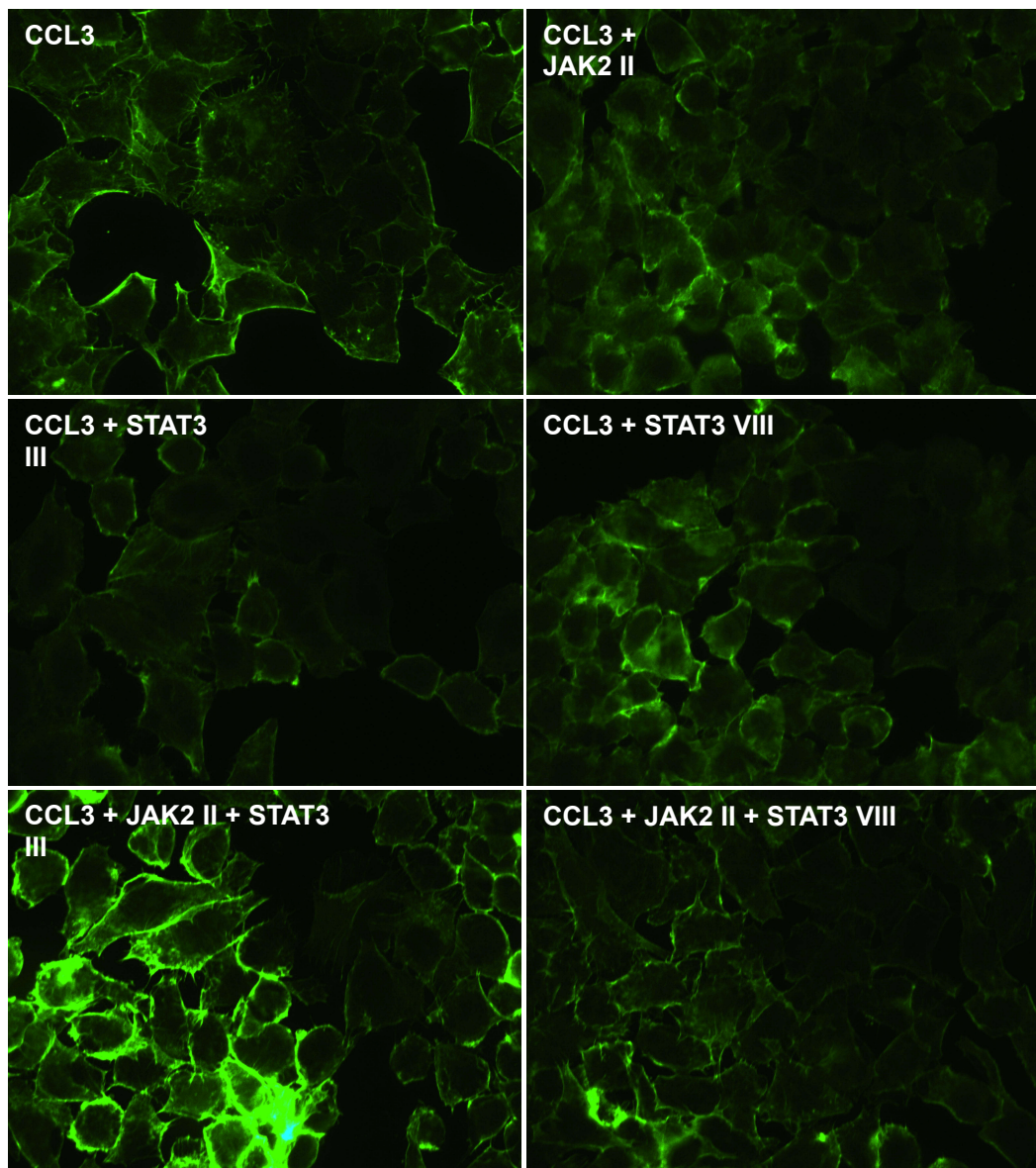


Figure 5.11: JAK2 inhibitor II, STAT3 inhibitors II and VIII and combinations do not affect actin polymerisation in HeLa.RC49. Micrographs of HeLa.RC49 monolayers treated with 100 nM CCL3 and JAK/STAT inhibitors. Cells were fixed and stained with Alexa-488 phalloidin actin stain (green). Images representative of population. Acquired with Leica imaging suite.

Since the THP-1 cells were capable of migration even in the presence of the Jak2/Stat3 inhibitors, we did not expect the inhibitors to have a similar disruptive effect on the actin cytoskeleton as cucurbitacin I. HeLa.RC49 were treated with JAK2 II, STAT3 III, STAT3 VIII and combinations of JAK2 II + STAT3 III and JAK2 + STATVIII for 0.5hr before activation for 1hr with 100 nM CCL3 and fixing and staining with Alexa-488 tagged phalloidin (Figure 5.11).

All treatments showed no pronounced differences from the control and there was no evidence of abnormalities in the actin cytoskeleton with the combined treatments. This provides further evidence that the effects observed with cucurbitacin I were not associated with its ability to inhibit JAK2/STAT3 signalling.

5.4. Discussion

The aim of this chapter was to determine if JAK2/STAT3 signalling played any role in CCR5 mediated signal transduction to chemotaxis. JAK/STAT signalling represents a well-researched oncogenic signalling axis in association with its canonical activation through cytokine receptors [249]. There is growing evidence that chemokine receptors [125, 252] and specifically CCR5 [40, 122] interact with and can activate JAK/STAT. Signalling through JAK2/STAT3 has been shown to be prevalent in numerous cancers and because CCR5 is known to activate JAK2 [122], the inhibitor of JAK2/STAT3 [126] cucurbitacin I was used to determine if JAK2 or STAT3 inhibition would disrupt the cytoplasmic signalling events associated with CCR5 activation. Cucurbitacin I had no effect on CCR5 mediated calcium release but reduced the migration in treated THP-1 cells to zero. As discussed earlier (chapter 3) the ability of an inhibitor to reduce migration to below basal levels warrants investigation to determine if a disruption of cytoskeletal structures has occurred. This is particularly pertinent because there is evidence that cucurbitacin I does disrupt actin dynamics in some cells [254]. To explore this idea and to determine if calcium mobilisation can occur with a disrupted actin cytoskeleton the actin polymerisation inhibitor [257] cytochalasin D was used to disrupt THP-1 cell actin formation before calcium flux and chemotaxis assay. Cytochalasin D treatment did not affect calcium release but completely blocked chemotaxis. Indeed the reduction was comparable to that seen with cucurbitacin I treatment. Phalloidin actin staining in HeLa.RC49 and CHO.CCR5 cells activated with CCL3 confirmed that cucurbitacin I was affecting actin polymerisation. Again the observed disruption was similar to that seen with cytochalasin D treatment. Disruption of actin fibers in THP-1 cells could not be determined by fluorescence microscopy due to a lack of resolution. A flow cytometry based method was validated as a technique for identifying actin-

disrupted THP-1 cells however the evidence provided by HeLa.RC49 and CHO.CCR5 was taken to be sufficient to draw conclusions about the effects of cucurbitacin on the cytoskeleton of cells. The data did not explain whether the effects of cucurbitacin I were a result of JAK2 or STAT3 inhibition or both combined or were due to non-specific effects of the inhibitor. To investigate this further, inhibitors specific for only JAK2 [127] and STAT3 [128, 255] were used to treat THP-1 cells. The JAK2 inhibitor II was unable to significantly affect CCL3 stimulated calcium release but both STAT3 inhibitors, while having no effect on EC₅₀ values, did reduce the level of stimulated calcium release in THP-1 cells and corresponded to significant reductions in calculated efficacy. STAT3 tyrosine 705 phosphorylation is required for translocation of the homodimerised protein to the nucleus [256]. A pTyr750 ELISA was used to validate if the inhibitor were blocking protein function; the assay revealed that JAK2 inhibition did not affect IL-6 stimulated STAT3 Tyr705 phosphorylation in THP-1. STAT3 inhibitors III and VIII blocked STAT3 Tyr705 phosphorylation in THP-1 and HeLa.RC49 cells suggesting that the reduction in calcium release could have been attributed to inhibition of STAT3. The failure of JAK2 inhibitor II to prevent STAT3 phosphorylation could be explained by redundancy in JAK/STAT signalling (Figure 5.12); 'canonical' IL-6R signalling through JAK1 to STAT3 would explain the lack of efficacy seen with JAK2 inhibitor II.

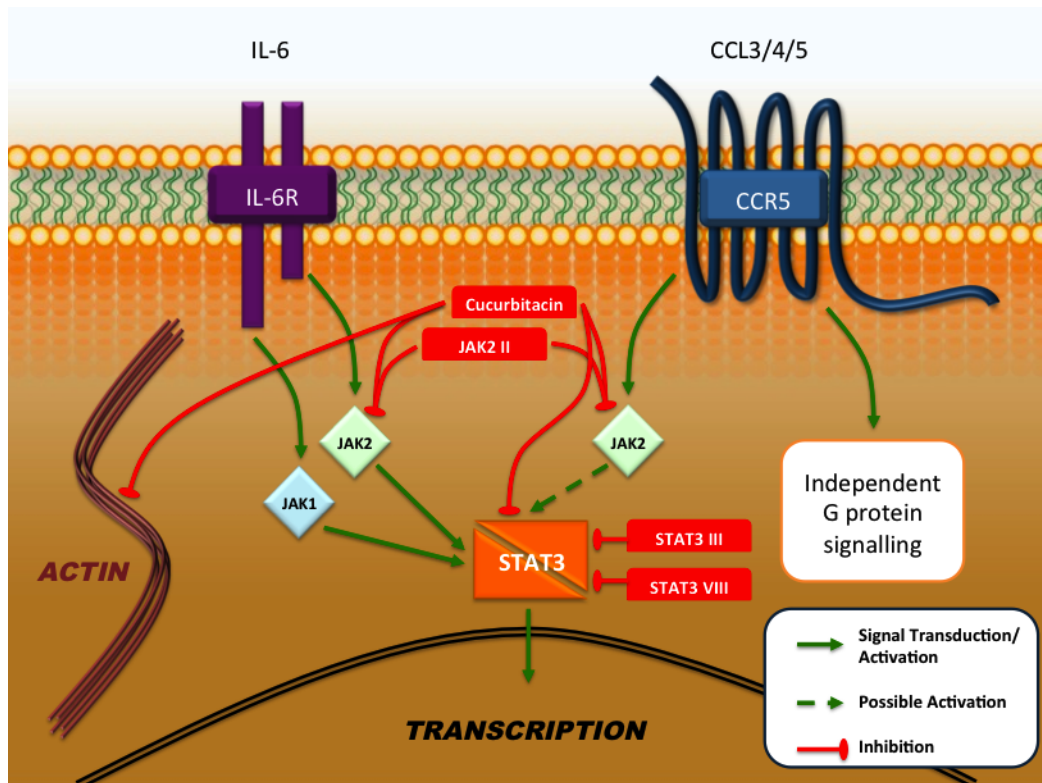


Figure 5.12: JAK/STAT signal transduction and inhibition explored in chapter 5. Activation of STAT3 via the interleukin 6 receptor (IL6-R) can occur via JAK1 or JAK2.

The effects of the two STAT inhibitors were less clear as cucurbitacin I had shown no significant effect on calcium release. There are no reports in the literature of STAT3 inhibition leading to a block with calcium release being shown to operate upstream of STAT3 activation in some signalling axes [258]. This suggested that either a novel signal transduction pathway was operating or the inhibitors were affecting THP-1 cells through 'off-target' mechanisms.

Assessment of the JAK2 and STAT3 inhibitors in THP-1 chemotaxis assays also produced conflicting data: JAK2 inhibitor 2 was shown to have no significant effect on migration which provided strong evidence that any JAK2 activity associated with CCL3 activated CCR5 was not involved in the cytoplasmic signal transduction to chemotaxis. Of the STAT3 inhibitors STAT3 III was able to significantly reduce migration whilst STAT3 VIII was not; as both inhibitors blocked Tyr705 phosphorylation this provided evidence that STAT3 phosphorylation was not required for migration but that the mode of binding of STAT3 III, which is different from STAT3 VIII [128, 255], may have been important. Pre-treatment with a combination of JAK2 II with STAT3 III and

STAT3 VII had no significant effect on migration, which showed that the dual inhibitory effect of cucurbitacin I was not responsible for actin disruption. This was supported by actin staining in HeLa.RC49 cells that showed no abnormalities in actin formation with combined JAK2/STAT3 inhibitor treatment. The observed effect of combined treatment on chemotaxis was confusing however because JAK2 II treatment in combination with STAT3 seemed to reverse the effects of STAT3 treatment alone. The only feasible explanation for this would be if JAK2 inhibitor II was interacting with STAT3 III directly, or with its binding site on STAT3 to prevent it functioning as an inhibitor however the lack of statistical difference between treatments does indicate an aberrant result may have occurred. Taken as a whole the data support the idea that JAK2/STAT3 signalling is not involved with CCR5 mediated chemotaxis at a cytoplasmic level; whether the increase in protein expression promoted by the JAK/STAT axis affects THP-1 migration over longer time periods was not explored, but fall into the canonical function of these proteins. As discussed in the previous chapter 4 the effects of the c-Src family non-receptor tyrosine kinase inhibitor bosutinib, which has been shown to inhibit STAT activation [79], are for this reason also not likely to be a result of STAT inhibition.

The discrepancy with STAT3 inhibitor III did require further investigation though. Using the results from this chapter as a basis Khabazzi *et al.* [47] investigated further the mechanism by which STAT3 inhibitor II affects CCR5 mediated migration. The conclusions of this investigation were that STAT3 III showed high levels of cytotoxicity over the time period of chemotaxis assays, which resulted in the decreased migration with none of the effects of the inhibitors attributable to cytoplasmic signalling to chemotaxis.

5.5. Chapter Conclusions

The final conclusions to be drawn from this chapter are:

- 1) JAK2/STAT3 inhibitor cucurbitacin I completely blocks chemotaxis in THP-1 by disrupting actin polymerisation.
- 2) Simultaneous inhibition with specific JAK2 and STAT3 inhibitors cannot replicate the detrimental effects on actin formation, which must, therefore, be attributed to 'off-target' effects specific to cucurbitacin I.
- 3) JAK2 inhibition has no effect on IL-6 induced STAT3 phosphorylation in THP-1, however JAK/STAT signalling redundancy may explain this observation.
- 4) JAK2/STAT3 play no transcription independent, cytoplasmic signal transduction role in CCR5 mediated chemotaxis in THP-1 cells, which supports the idea that the effects of bosutinib are not likely to be due to STAT inhibition.

CHAPTER 6: A pharmacological investigation of internalisation machinery: matching membranes to migration

6.1. Introduction

Chapter 4 suggests that signalling from the CCR5/CCR1 receptors to chemotaxis operates through an arrestin dependent mechanism. Other signalling points were also identified, including non-receptor tyrosine kinases and the Rho kinase (ROCK). Inhibition of these two proteins results in a blockade of migration indicating that they represent points of pharmacological access to CCR5 mediated migration. These findings also left some questions unanswered: one of them being whether internalisation itself is required for migration. The importance of internalisation may be an alternative explanation for the observations made using PKC and GRK2 inhibitors. The exact signalling partners of non-RTKs and ROCK were also not confirmed, as was the question, whether PI3K function is required in CCR5 mediated migration. This is of particular importance considering the conflicting reports in the literature [115, 248]. This chapter will attempt to address some of these questions by investigating the role of receptor internalisation and endocytosis on migration by direct blockade of the process and by inhibition of the machinery of endocytosis identified in the introduction. Proteins such as clathrin and dynamin are of particular interest because they have been shown to interact with arrestins [64], G $\beta\gamma$ [107], phosphoinositides [259] and Rho kinases [192] and have links to migration [77, 188]. The role of non-RTKs will also be investigated by examining the role of SH2 domain containing adaptor proteins identified in the literature. These experiments will allow the relationships of the different signalling stations identified in chapter 4 to be resolved and will potentially lead to the identification of novel targets for the inhibition of migration.

6.2. Chapter Aims

Initially the role of internalisation in endocytosis will be determined by small molecule inhibition of clathrin and dynamin. A range of chemically diverse dynamin inhibitors are available which allow the resolution of specific dynamin interactions which could be important in THP-1 migration. The adaptor protein Grb2 has links with dynamin, CCR5 and migration so the role of this protein will be investigated through small molecule inhibition as a possible link between non-RTK tyrosine phosphorylation and cytoskeletal machinery.

6.3. Results

6.3.1. CCR5 mediated endocytosis is not required for migration in THP-1 cells

Chapter 4 showed that overexpression of arrestin 2 and 3 results in a decrease in CCR5 induced calcium release that was assumed to be a consequence of increased receptor desensitisation and internalisation. The effect of arrestin overexpression in THP-1 migration could not be experimentally determined due to adverse effects of the electroporation transfection process. Inhibition of GRK2 and PKC in THP-1 cells, which can recruit arrestins to activated GPCRs, revealed potentially divergent roles of arrestin in CCR5 signalling to migration, which is thought to be based on their mode of recruitment to the CCR5 Carboxyl-terminus (Chapter 4). It was not clear however whether the effects of the inhibition of GRK2 and PKC were due to decreases in arrestin mediated signalling or increases in internalisation and desensitisation. The increases in migration seen with PKC inhibitor treatment was theorised to be a result of alterations to arrestin signalling bias, however, because the effect on internalisation was not measured an increase or decrease in endocytosis may also have been responsible. Investigation into whether receptor endocytosis is required for THP-1 cell chemotaxis will allow a further clarification of the role of arrestins. Endocytosis inhibitors were used to investigate whether internalisation is a key requisite in the migration of cells, even though these processes are thought to occur independently of the formation of arrestin dependent signalling complexes [35]. CCR5 receptor

internalisation has been shown to occur through clathrin and caveolin dependent pathways [213]. Clathrin dependent endocytosis can be inhibited by hyperosmolar sucrose solutions [178] and caveolae are sensitive to cholesterol disruption through filipin treatment [74]. It was elected to treat THP-1 with a hyperosmolar sucrose and a combination of hyperosmolar sucrose and filipin to block both forms of internalisation. The clathrin endocytosis inhibitor sucrose was used on its own and in conjunction with the caveolae specific inhibitor filipin (Figure 6.1a). The cell permeable clathrin inhibitor Pit stop 2 was also used (Figure 6.1b).

Treatment with hyperosmolar sucrose did not affect THP-1 migration toward 1 nM CCL3 ($p > 0.05$ vs. control) nor did treatment with hyperosmolar sucrose and filipin combined ($p > 0.05$, $n = 3$) (Figure D1a). These data indicate that endocytosis is not required for functional migration in THP-1 cells. The non-significant trend towards increased migration with the combined sucrose and filipin was also interesting as it suggested that stalling the internalisation process enhanced migration, possibly by allowing prolonged arrestin signalling. To determine if these observation can be reproduced from 'the inside out' via cytoplasmic inhibition of clathrin, the recently described inhibitor of clathrin coated pit formation pit stop 2 [260] was used to treat THP-1 cells (Figure 6.1b). Pit stop 2 demonstrated an ability to increase migration in THP-1 cells: 1.25 μM treatment showed no significant effect ($p > 0.05$, $n = 6$ vs. negative control compound) but 30 μM (the recommended treatment level [260]) increasing THP-1 cell migration towards 1 nM by $30 \pm 4\%$ (vs. negative control adjusted for basal migration). These data support the non-significant observation seen with sucrose + filipin treatment and suggest that maintaining activated CCR5 at the cell surface increases signalling to chemotaxis. This combined with the data from chapter 4 suggest that arrestin dependent signalling formed an important component of CCR5 signalling axis to chemotaxis.

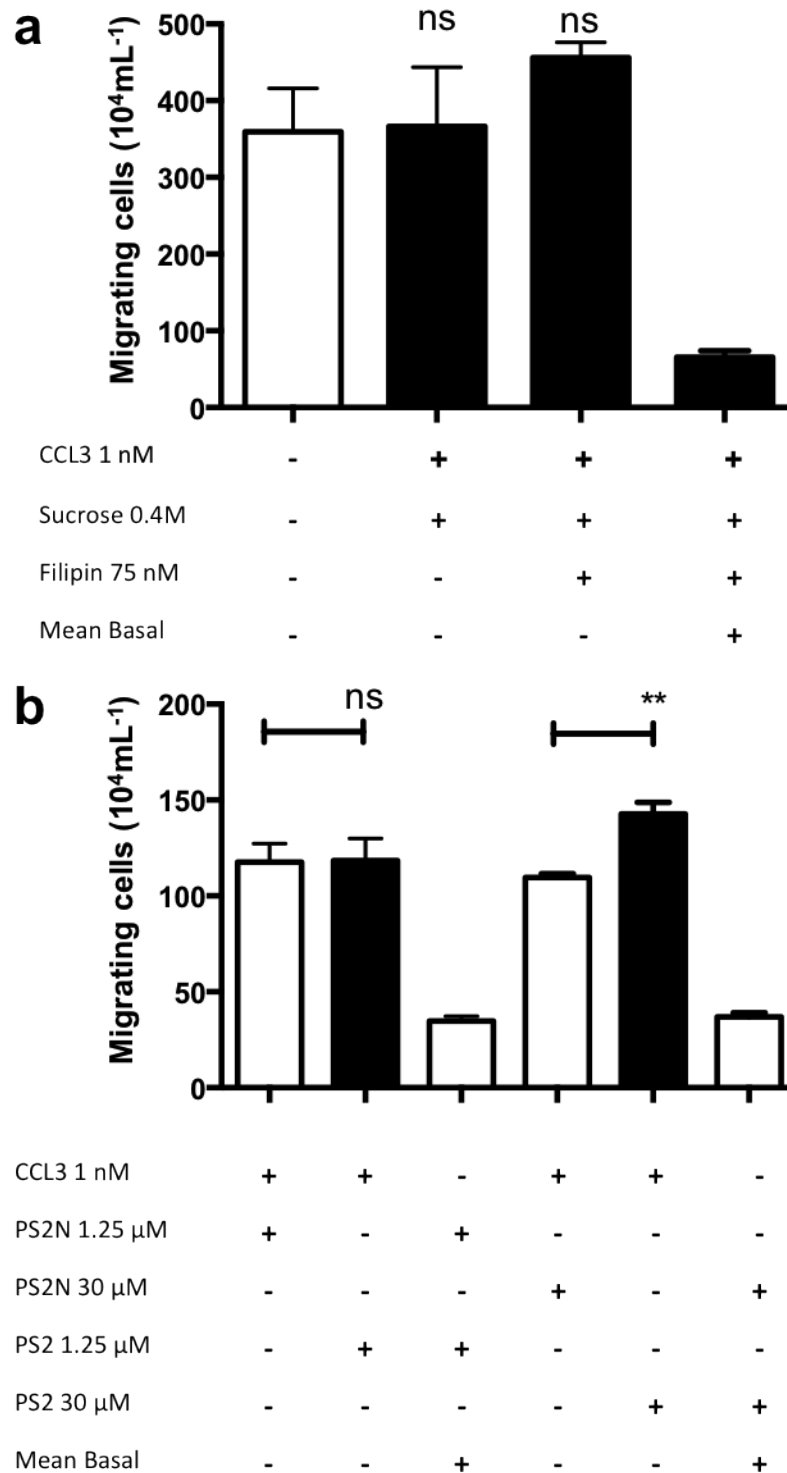


Figure 6.1: Inhibition of CCR5 internalisation does not reduce CCL3 stimulated chemotaxis in THP-1 cells. (a) Treatment with a hyperosmolar sucrose solution and sucrose + fillipin has no significant effect on CCR5 mediated chemotaxis in THP-1 cells (n=3). (b) Treatment of THP-1 cells with the inhibitor of clathrin mediated endocytosis, Pit stop 2 (30 μ M), significantly increases migration towards 1 nM CCL3 by 30% \pm 4 (p 0.01 n=6) vs. negative control compound

(PS2N). Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. ** = $p \leq 0.01$ vs. vehicle control, ns = not significant).

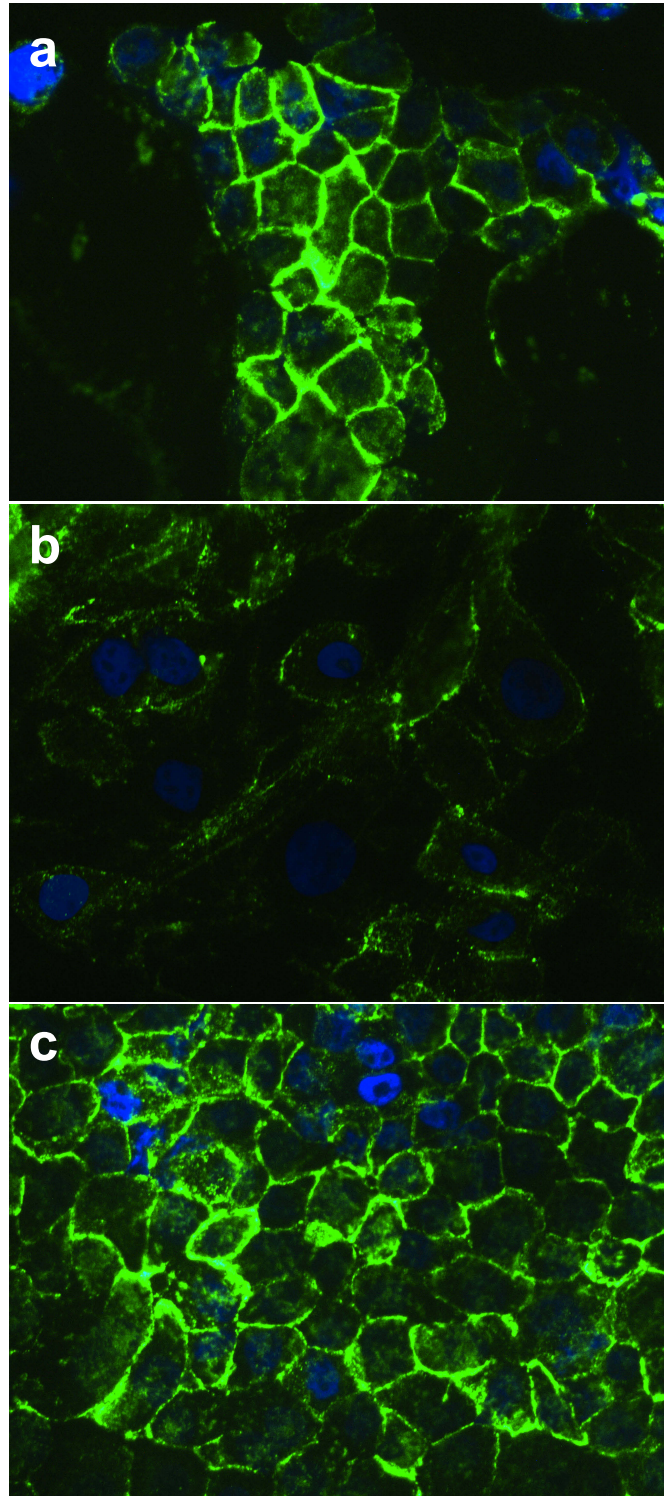


Figure 6.2: Pit stop 2 prevents CCR5 internalisation in HeLa.RC49 cells. (a) Negative control cells treated with anti-CCR5 Ab and Alexa-488 tagged secondary Ab. (b) Addition of 100 nM

CCL3 for 1hr promotes CCR5 internalisation. (c) Pit stop 2 prevents CCL3 stimulated internalisation. (Nuclear stain with DAPI (blue), images representative of population. Acquired with Leica imaging system)

The inhibitory effects of pit stop 2 were validated through the visualisation CCL3 induced internalisation of CCR5 in HeLa.RC49 after treatment with pit stop 2. (Figure 6.2). Untreated control cells demonstrated significant amounts of membrane bound CCR5 as demonstrated by a continual ring of fluorescence around the periphery of the cell. Upon CCL3 stimulation there was a marked reduction in surface expressed CCR5 as demonstrated by a reduction in the continuity of the fluorescent 'ring'. Cells pre-treated with pit stop 2 were comparable to the untreated, unstimulated control cells. These data indicate that internalisation of activated CCR5 receptors is indeed inhibited by pit stop 2 in HeLa cells, which shows that clathrin dependent internalisation of CCR5 is not likely to be required for CCL3 induced THP-1 cell migration.

In the introduction the conflicting evidence for the role of PI3K in migration was discussed. Whilst it has been shown that PI3K is crucial for migration [57, 95, 248] and is activated by CCR5 as part of the arrestin scaffold [39] there is also evidence that monocytes such as THP-1 cells do not require PI3K mediated polarisation for migration [115].

PI3Ks have several isotypes which show significant differences in recruitment and functionality [78] with PI3K γ , and to a lesser extent PI3K β associated with GPCR signalling via G $\beta\gamma$ activation [110, 114, 216]. The pan isotype PI3K inhibitor LY294002 is well characterised [116, 261] and is known to inhibit migration in numerous systems at concentrations around 10 μ M [248].

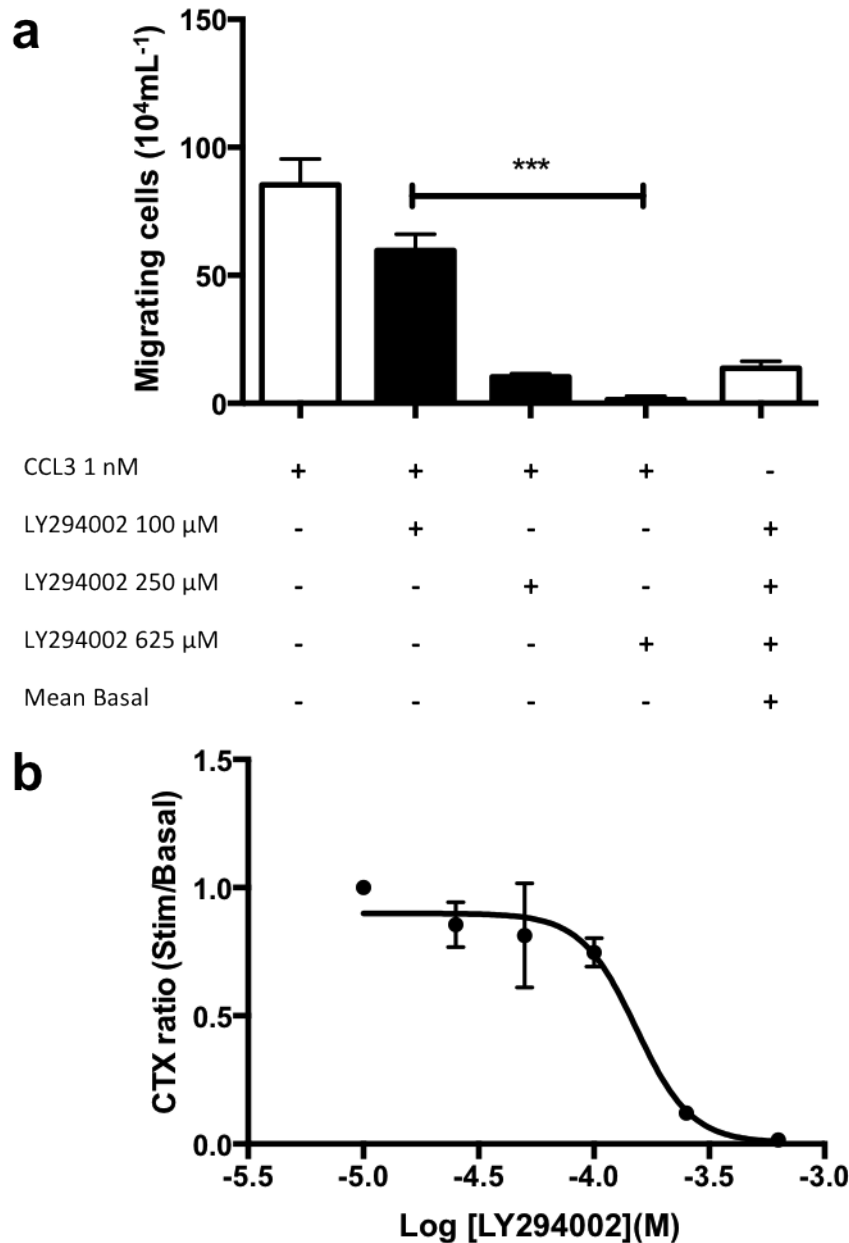


Figure 6.3: CCL3 stimulated chemotaxis in THP-1 cells is only sensitive to pan-isotype PI3K inhibitor LY294002 at very high concentrations. (a) Migration in THP-1 cells can be significantly reduced with concentrations of reversible pan-isotype PI3K inhibitor LY294002 at and above 100 μ M ($p=0.001$, $n\geq 3$). (b) Concentration response of for LY294002 inhibition of THP-1 chemotaxis ($n\geq 3$). All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. *** indicates $p\leq 0.001$ vs. vehicle control).

THP-1 cells were pre-treated with LY294002 at concentrations between 25 and 625 μM to determine the effect of PI3K inhibition on CCL3 stimulated THP-1 migration (Figure D3a). LY294002 has no significant effect on THP-1 migration at 25 and 50 μM treatments, with significant reductions in migration only seen after 100 μM treatment (Figure 6.3a). The potency of LY294002 in this assay was calculated by fitting a sigmoidal concentration response curve (with an assumed Hill coefficient of 1) which produced an $\text{IC}_{50} = 152 \pm 13 \mu\text{M}$, which is significantly higher than the calculated value for PI3K inhibition [116] and normal inhibition of chemotaxis [248]. The potency level also suggested that secondary targets of LY294002 such as PIM1 for which it shows an $\text{IC}_{50} 4 \mu\text{M}$ [261] might be targeted. These data suggest that PI3K is not important for THP-1 cell migration. To clarify whether there is a role for PI3K in CCL3 induced migration the non-reversible pan-isotype PI3K inhibitor wortmannin and the PI3K γ specific inhibitor AS605240 were also used to treat THP-1 cells.

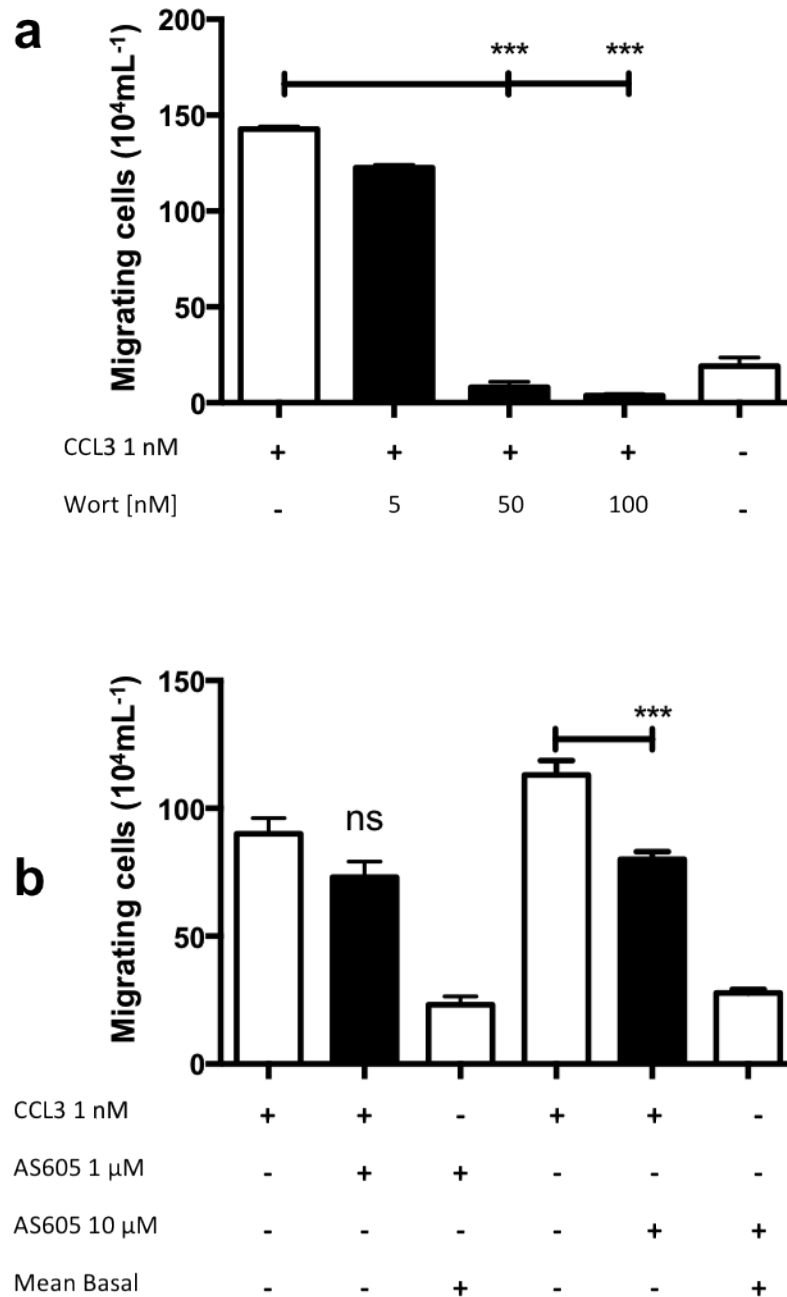


Figure 6.4: THP-1 cell migration towards CCL3 is sensitive to pan-isotype PI3K inhibitor wortmannin but not PI3Kγ inhibitor AS605240. (a) Irreversible PI3K inhibitor wortmannin (Wort) significantly reduces THP-1 chemotaxis at 50 nM ($p \leq 0.001$ $n=3$). (b) PI3Kγ specific reversible inhibitor AS605240 (AS605) reduces migration at 10 μM ($p \leq 0.001$ $n=3$). All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison.*** indicates $p \leq 0.001$ vs. vehicle control).

Wortmannin is a highly potent, non-reversible inhibitor of all human PI3ks with an IC_{50} of 1 nM [117]. Pre-treatment of THP- cells with 5, 50 and 100 nM wortmannin revealed that although significant reductions in migration were seen at 500 and 100 nM ($p \leq 0.001$, $n=3$) there was no significant reduction in migration seen with 5 nM treatment (Figure 6.4a). As with LY294002, wortmannin has a secondary target, polo like kinases [262], for which it shows inhibitory potency of around 25 nM [263]. These data show that wortmannin inhibitory concentrations are higher than expected for effects due to PI3K alone and fall into concentrations where secondary target inhibition has to be considered. AS605240 is a rationally designed PI3K inhibitor described as 'specific' for PI3K γ due to the fact that its IC_{50} for PI3K γ of 5 nM is 7.5 times lower than the α -isotype and 30 times lower than the δ and β -isotypes [118]. AS605240 has been shown to effectively inhibit PKB phosphorylation in THP-1 cells signalling through GPCRs and has no known secondary targets [216]. The use of increasing inhibitor concentration from low nanomolar upwards should allow for a determination of functional PI3K isotypes which are present in CCL3 stimulated THP-1 cells. However, treatment of THP-1 cells with AS605240 from 1-500 nM had no significant effect on migration (data not shown). At 1 μ M, 6 fold higher than the IC_{50} for the least specific PI3K isotypes, AS605240 continued to show no significant effect on THP-1 migration towards 1 nM CCL3 (Figure 6.4b) ($p > 0.05$, $n=6$). Treatment with AS605240 at 10 μ M, 5000 fold higher than the reported PI3K γ IC_{50} , showed a significant ability to reduce THP-1 migration towards CCL3 ($p \leq 0.001$, $n=3$), however, this reduction was only partial, and considering the high inhibitor concentration, did not provide convincing evidence that PI3K inhibition was responsible for the observation. The evidence that PI3Ks, classical mediators of migration, appeared not to be involved which raises some interesting questions but supports the work of Volpe *et al.*

Clathrin is known to interact with a number of other proteins, such as arrestin and dynamin, which have strong links to migration outside of their 'classical roles' as mediators of endocytosis [180, 188, 264]. In recent years a large number of chemically diverse dynamin inhibitors have been described (Table 1.3) [158, 204, 205, 209]. These inhibitors, whilst initially identified and developed for their efficacy to inhibit dynamin GTPase, also showed varying abilities to inhibit

receptor mediated endocytosis (RME), stabilise dynamin polymers [202] and to target different regions of the dynamin protein (Figure 1.3.). The role of dynamin itself in migration is not well understood and therefore the effect of the panel of inhibitors on THP-1 migration was investigated

6.3.2 The effect of dynamin inhibition on C-C motif chemokine receptor mediated chemotaxis

As discussed in the introduction the dynamin 1 and 2 GTPase inhibitor dynasore was the first small molecule dynamin inhibitor that was discovered. The inhibition of dynamin GTP turnover prevents the 'pinching' motor function of dynamin and prevents scission of clathrin-coated vesicles however the binding of inhibitors to protein also has the ability to affect the way the protein can interact with the cytoplasmic milieu. The effects of dynasore on THP-1 migration were therefore determined. Based on the observations with pit stop 2 and sucrose it can be expected, that dynasore treatment will have either no effect or indeed increase migration by prolonging the signalling if dynamin is solely acts as part of the endocytosis machinery. There is, however, also a large body of evidence that demonstrates a role for dynamin outside of endocytosis as a mediator of actin dynamics [77, 100, 265].

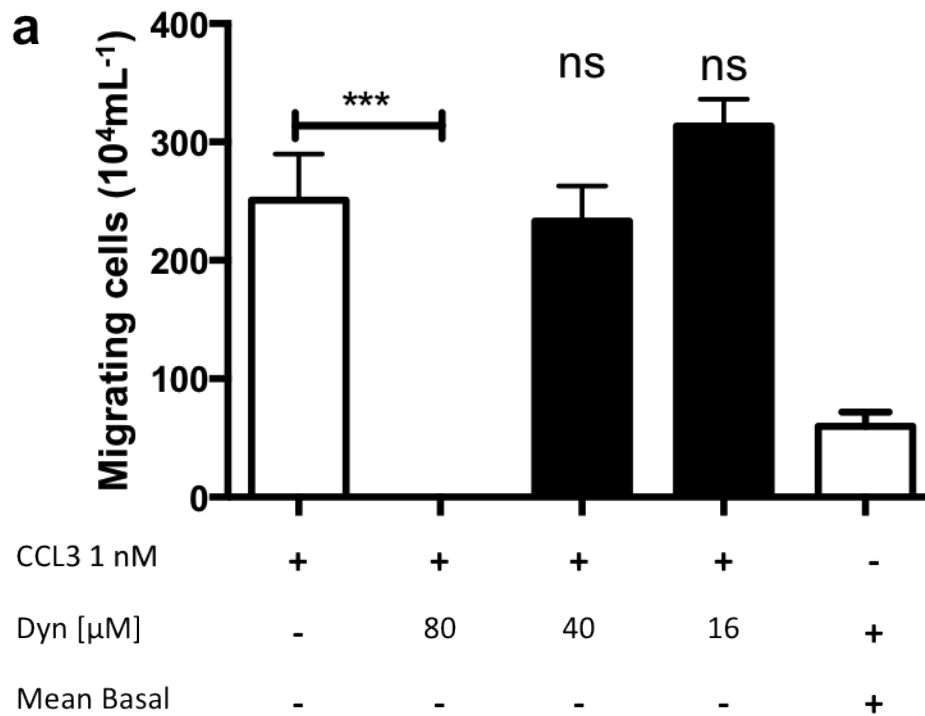


Figure 6.5: Dynasore prevents CCR5 mediated chemotaxis in THP-1 cells. (a) The dynamin GTPase inhibitor dynasore (Dyn) reduces THP-1 migration towards 1 nM CCL3 to sub-basal levels vs. vehicle control ($p=0.001$ $n=6$). All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.001$ vs. vehicle control ns = not significant).**

Pre-treatment of THP-1 with dynasore significantly reduced THP-1 cell migration towards CCL3 ($p \leq 0.001$, $n=6$ vs. vehicle control) to sub-basal levels (Figure 6.5). This inhibition occurred only over a narrow range of concentration, with treatment at 40 and 16 μM showing no significant reduction in chemotaxis ($p > 0.05$, $n=6$). To determine if dynasore was functioning as an inhibitor of CCR5 internalisation, HeLa.RC49 cells were used to visualise internalisation in the presence of the inhibitor.

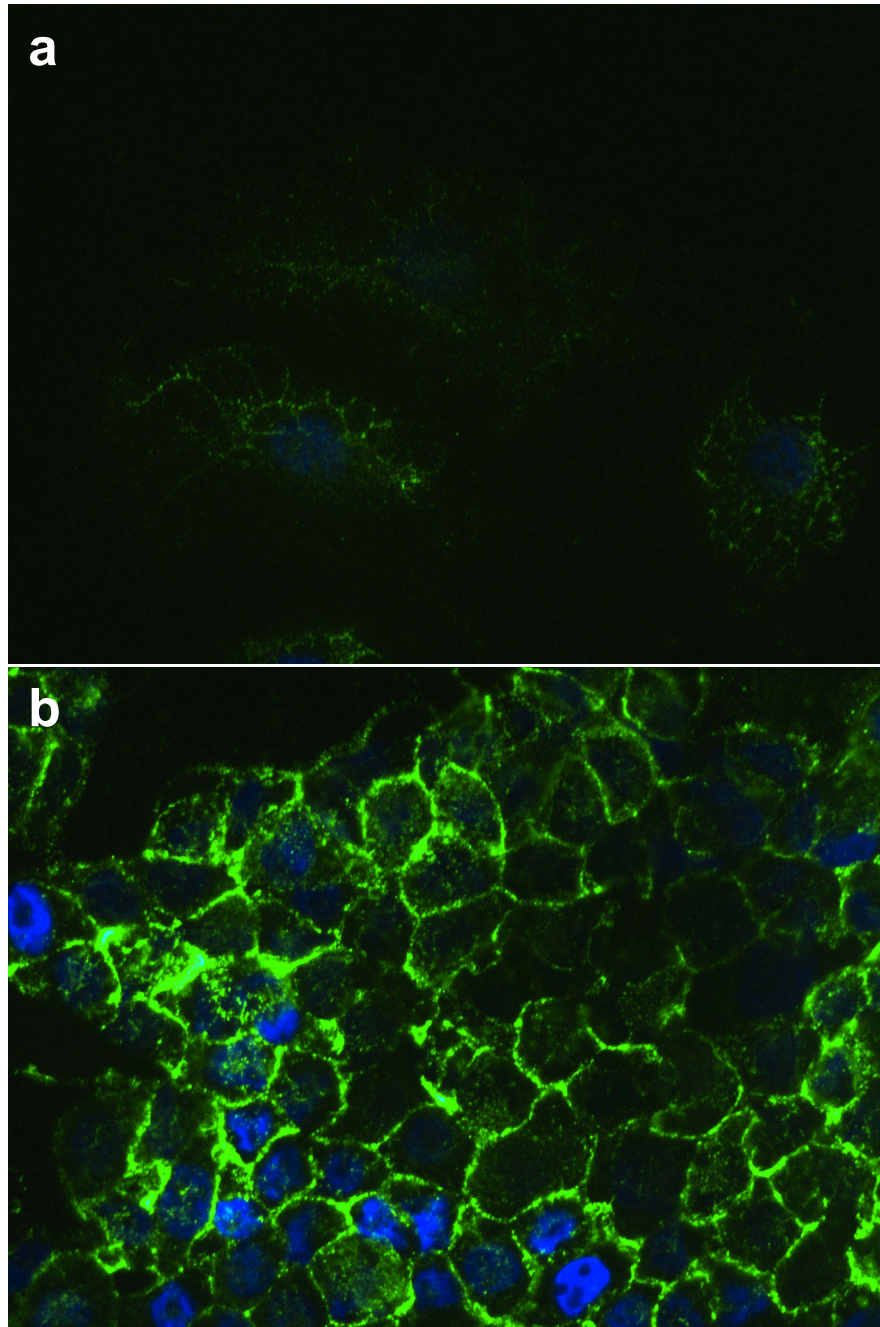


Figure 6.6: Dynasore prevents CCL3 stimulated internalisation of CCR in HeLa.RC49 cells. (a) HeLa.RC49 cells stimulated with 100 nM CCL3 for 1hr show an increase in receptor internalisation when compared to (b) cells treated for 0.5hr with 80 μ M Dynasore. (Anti-CCR5 Ab counterstained with Alexa-488 tagged Ab, DAPI nuclear stain). Images are representative of the population. Acquired with Leica imaging system.

HeLa.RC49 stimulated with 100 nM CCL3 showed a marked reduction in surface expression of HEK1/85/7a Ab stained CCR5 (Figure 6.6). Pre-treatment of HeLa.RC49 with 80 μ M dynasore before CCL3 stimulation resulted in significantly more surface expressed receptors compared to the untreated control cells. These results indicated that dynasore did indeed inhibit endocytosis, but based on the results seen with sucrose/flipin and pit stop these results were contradictory, and suggested that other functions of dynamin may have been responsible. Despite the evidence that calcium flux and chemotaxis are independent in THP-1 cells, the effect of dynasore on CCL3 stimulated calcium release was determined to provide a broader picture of dynamin dependent signalling events.

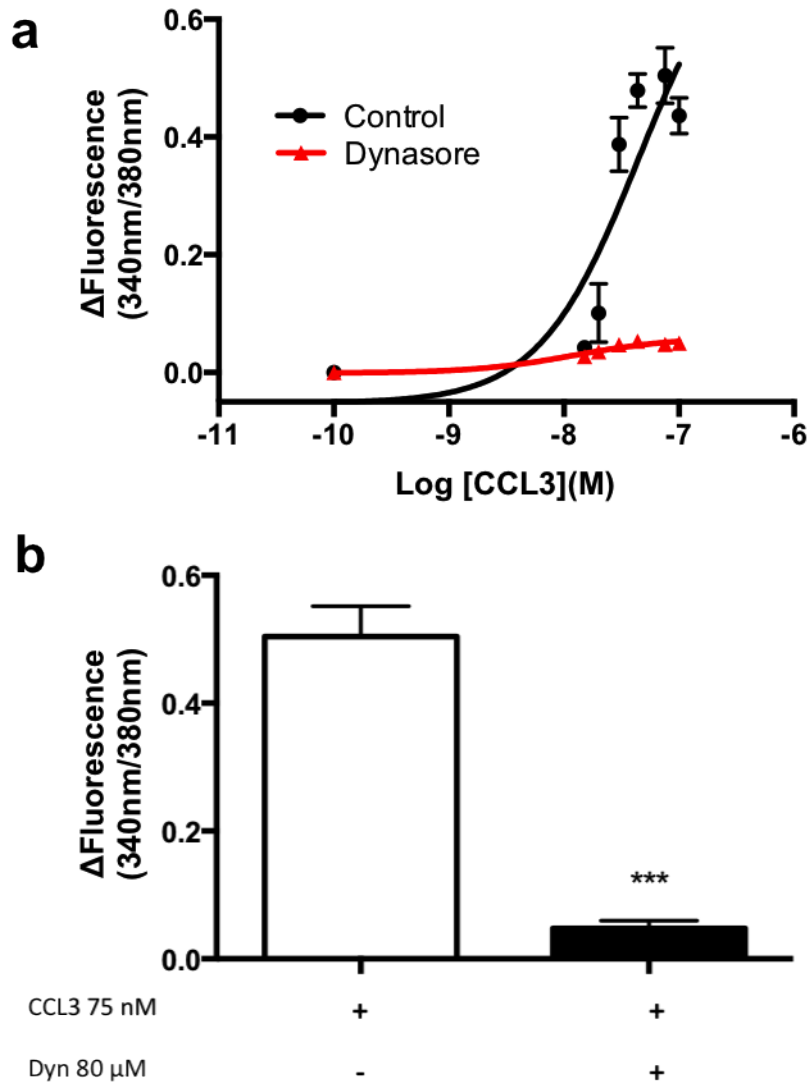


Figure 6.7: Dynasore inhibits CCL3 stimulated calcium flux in THP-1 cells. (a) Concentration response for calcium release in THP-1 stimulated by CCL3 in cells treated for 0.5hr with 80 μ M dynasore vs. control ($n \geq 3$). (b) Calcium flux in THP-1 stimulated by 75 nM CCL3 is significantly reduced by treatment with 80 μ M dynasore ($p \leq 0.001$ $n \geq 3$). Results represent the mean \pm SEM of at least 3 independent experiments (Unpaired t-test. *** indicates $p \leq 0.001$ vs. vehicle control).

Pre-treatment of THP-1 with 80 μM dynasore demonstrates that the inhibitor has a marked effect on the ability of CCL3 to induce calcium release (Figure 6.7a). Efficacy for dynasore treated cells could not be predicted due to the inability to fit a sigmoidal concentration response curve, however calcium mobilisation stimulated by 75 nM CCL3 was significantly reduced ($p \leq 0.001$, $n=3$ vs. control) to almost negligible levels. Despite HeLa.RC49 visualisation indicating that cytotoxicity is not a major issue, the obvious interpretation of the observations made for THP-1 treatment with dynasore was that a reduction in cell viability could be responsible for the lack of signalling.

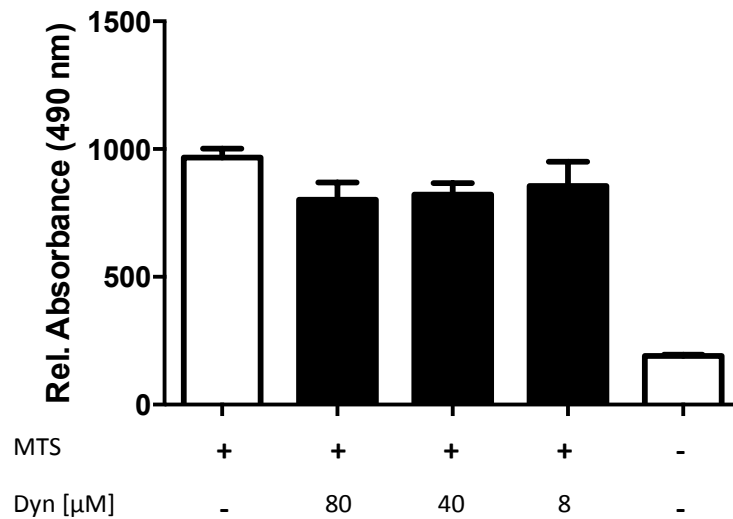


Figure 6.8: Dynasore shows no cytotoxicity in THP-1 after 5h exposure. There is no significant reduction in viability in THP-1 treated with dynasore for 0.5hr then incubated with MTS for 5hr ($n=3$). Results represent the mean \pm SEM of at least 3 independent experiments. Results represent the mean \pm SEM of at least 3 independent experiments vs. vehicle control.

The MTS cytotoxicity assay was performed on THP-1 treated with varying concentrations of dynasore over a time period equivalent to that during chemotaxis assay (Figure 6.8). Dynasore concentrations of 80, 40 and 8 μM did not significantly affect THP-1 viability ($p > 0.05$, $n=3$ vs. untreated control) which showed that the effect of dynasore on THP-1 cellular responses can not be attributed to cell death and will need to be explained in terms of the cellular functions of dynamin.

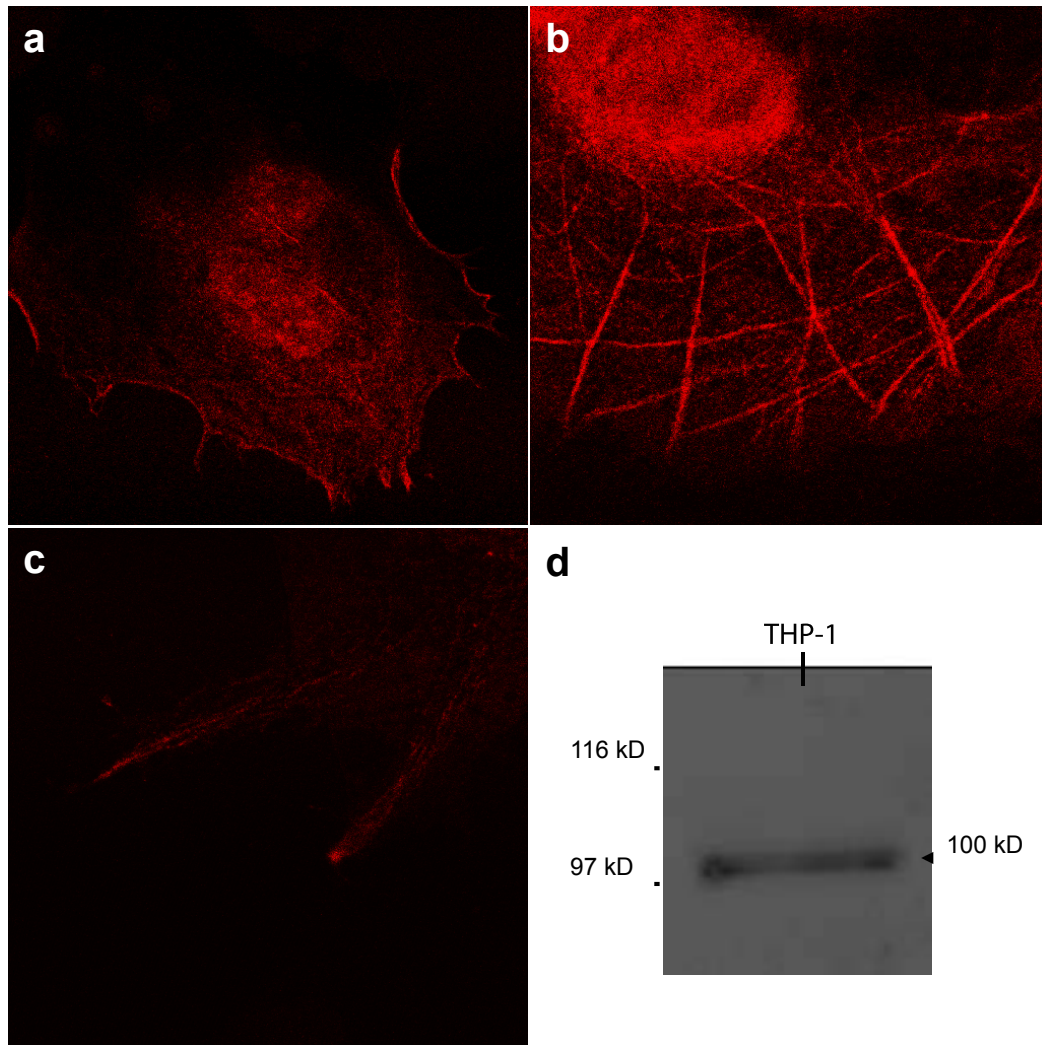


Figure 6.9: Dynamin localises at cytoskeletal structures in CHO.CCR5 and is present in THP-1 cells. (a) Confocal microscopy revealed that dynamin localises around the nucleus and at the leading edges of CCL3 stimulated CHO.CCR5. (b) Localisation with tubular structures is also observed. (c) Localisation with fibrous structures in filopodia is also observed. (d) Western blotting confirms the 100 kD protein dynamin 2 is present in THP-1 cells. Images obtained with Zeiss laser scanning confocal microscope system.

Although dynamin 2 is ubiquitously expressed it is important to validate the presence of dynamin 2 in the model cell lines before further conclusions can be drawn about the effect of dynasore. CHO.CCR5, which were used for visualisation of cytoskeletal structures, were stained with anti-dynamin 2 polyclonal Ab and with Alexa-594 tagged secondary Ab. Confocal microscopy and various z-axis slices demonstrated localisation of dynamin (verified with

secondary Ab control, not shown) with various structure including lamellapodia (Figure 6.9a), tubular structures reminiscent of microtubules (Figure 6.9b) and fibers and the terminal regions of filopodia (Figure 6.9c). These observations are supported by the literature [163, 164]. THP-1 cells are not conducive to imaging due to their small size, but western blotting was used to demonstrate a protein identified with rabbit polyclonal anti-dynamin 2 antibody at around 100 kD which corresponds with dynamin 2. This observation again is supported by the literature [178].

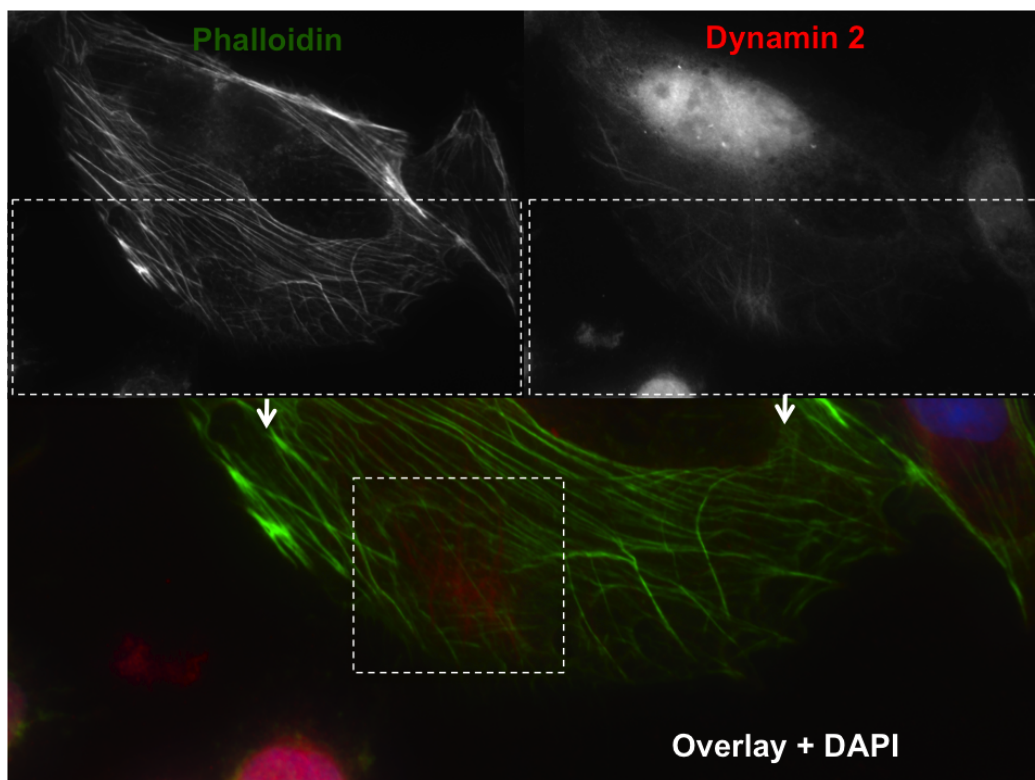


Figure 6.10: Dynamin localisation is prevalent along tubular structures and at the terminal regions of actin stress fibers. CHO.CCR5 activated with CCL3 (100 nM, 0.5hr) and stained with anti-dynamin 2 Ab counterstained with Alexa-594 tagged secondary Ab (red). Actin stained with Alexa-488 tagged phalloidin (green) and DAPI nuclear stain (blue). Image is representative of population. Acquired with Zeiss axiovision 2 system.

Disruption of the actin cytoskeleton is another obvious explanation for the observed effect of dynasore on THP-1 cell migration although this will not explain the observed reduction in calcium release. CHO.CCR5 cells treated with 80 μ M dynasore and stained for dynamin 2 and actin at the same time showed no disruption of actin polymerisation as seen with cytochalasin D (Chapter 5). Dynasore did not appear to affect dynamin localisation with the tubular structures that did not stain with phalloidin (Figure 6.10). These tubular structures were thought to be microtubules as dynamin interaction with microtubules is well characterised and is thought to be responsible for some of the effects of dynamin inhibitors on cancer cell proliferation [163, 266]. As microtubules have also been linked with migration [267] the microtubule polymerisation inhibitor nocodazole was used to detect whether dynamin modulation of microtubule polymerisation was important for the migration of THP-1 cells.

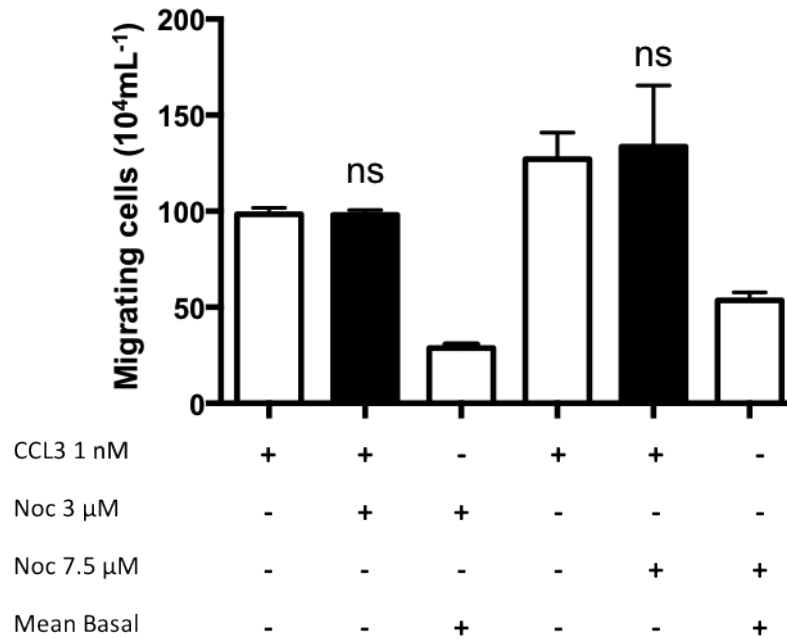


Figure 6.11: Microtubule polymerisation inhibitor does not inhibit THP-1 cell chemotaxis. Treatment of THP-1 cells with 3 and 7.5 μM nocodazole (Noc) for 2hrs prior to chemotaxis assay shows no significant effect on THP- migration towards 1 nM CCL3 (One-way ANOVA, Bonferroni multiple comparison. $n=3$, ns=not significant $p>0.05$).

Pre-treatment of THP-1 cells with 3 and 7.5 μM nocodazole prior to chemotaxis assay shows in no significant alterations to the way THP-1 cells respond ($p>0.05$, $n=3$ vs. vehicle controls) (Figure 6.11). Nocodazole pre-treatment for 12-18hrs is typically employed for disruption of the entire microtubule network however treatment for 1hr at low μM levels has been shown to inhibit additional microtubule polymerisation of existing microtubules [268]. These data suggest that microtubule polymerisation post stimulus is not required for CC motif chemokine receptor mediated chemotaxis in THP-1 cells.

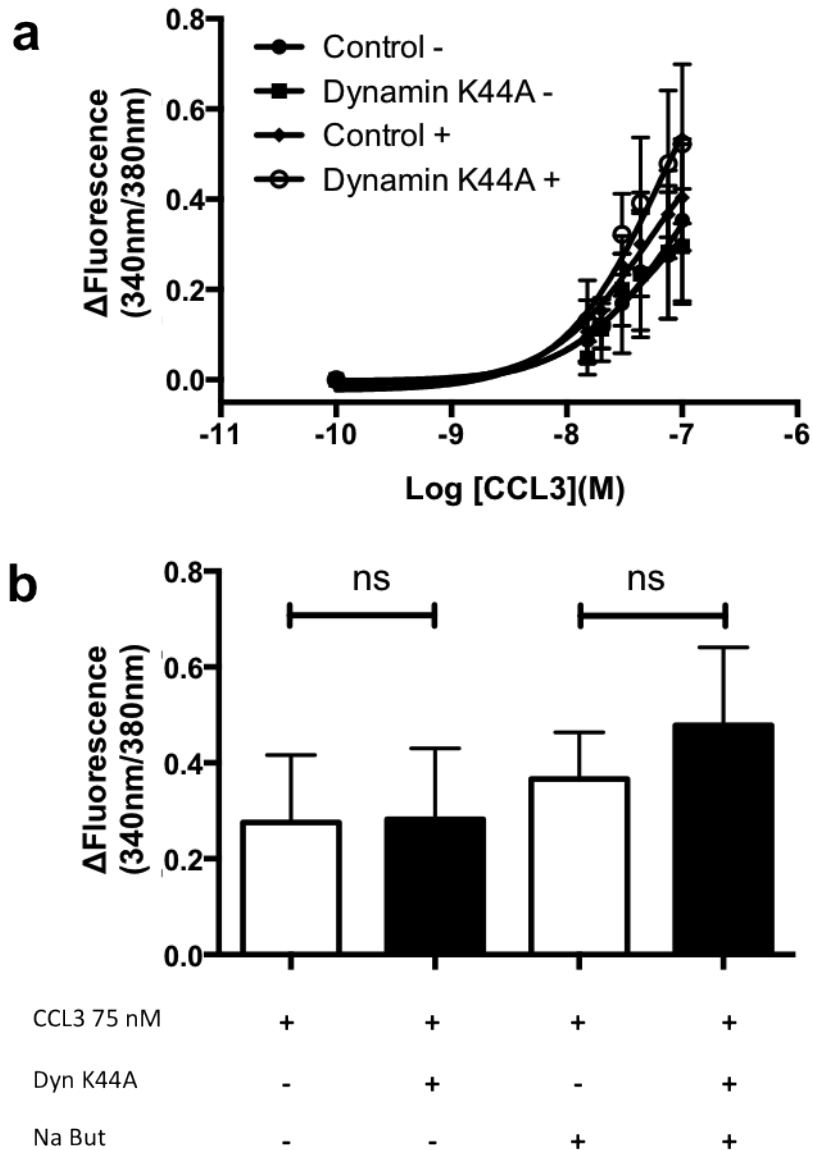


Figure 6.12: Transient transfection with dominant negative mutant dynamin K44A does not significantly affect CCR5 mediated calcium release. (a) Concentration response for calcium release in THP-1 stimulated by CCL3 in cells transfected with dynamin K44A plasmid (controls electroporated without plasmid) with (+) and without (-) sodium butyrate (Na But) (Results represent the mean \pm SEM of 2 independent experiments). (b) Calcium release stimulated by 75 nM CCL3 is not significantly different in dynamin K44A transfected cells (n=2)(Unpaired Student's t-test, ns = not significant).

THP-1 are sensitive to electroporation transfection in such a way that migration is disrupted; calcium release however, is unaffected by this procedure. The dominant negative dynamin 2 mutant K44A was used to investigate whether the GTPase inhibitory activity of dynasore could be attributed to dynamin GTPase function on calcium release stimulated through CCR5. Dynamin K44A shows no ability to catalyse GTP turn over and has been shown to reduce migration in cells over expressing the protein [149]. THP-1 cells were transfected with dynamin K44A plasmid or exposed to the transfection procedure without plasmid DNA as a control. Sodium butyrate was also used in an attempt to enhance plasmid protein expression (Figure 6.12). Both transfected and non-transfected cells demonstrated high variability in calcium release in stimulation with CCL3 and there was certainly no correlation between dynamin K44a transfected cells and the reduced calcium release seen with dynasore.

6.3.3. Pharmacological assessment of dynamin inhibitors on CCL3 mediated THP-1 migration

The breadth of recently described dynamin GTPase inhibitors is discussed in the introduction; these inhibitors have a range of different GTPase and RME inhibition potencies, different dynamin binding sites and diverse effects on migration (Table 1.3.). The systematic treatment of THP-1 cells with these inhibitors should allow the function of dynamin in chemotaxis to be clarified. The systematic inhibition of dynamin was started with the long-chain ammonium salts MiTMAB and OctMAB. MiTMAB and OctMAB have been shown to inhibit dynamin GTPase function and RME and to function through interaction with the dynamin pleckstrin homology domain [203, 204]. The negative control compound pro-mystric acid shares structural homology with MiTMAB and OctMAB but has no function against dynamin GTPase or endocytosis functions [203].

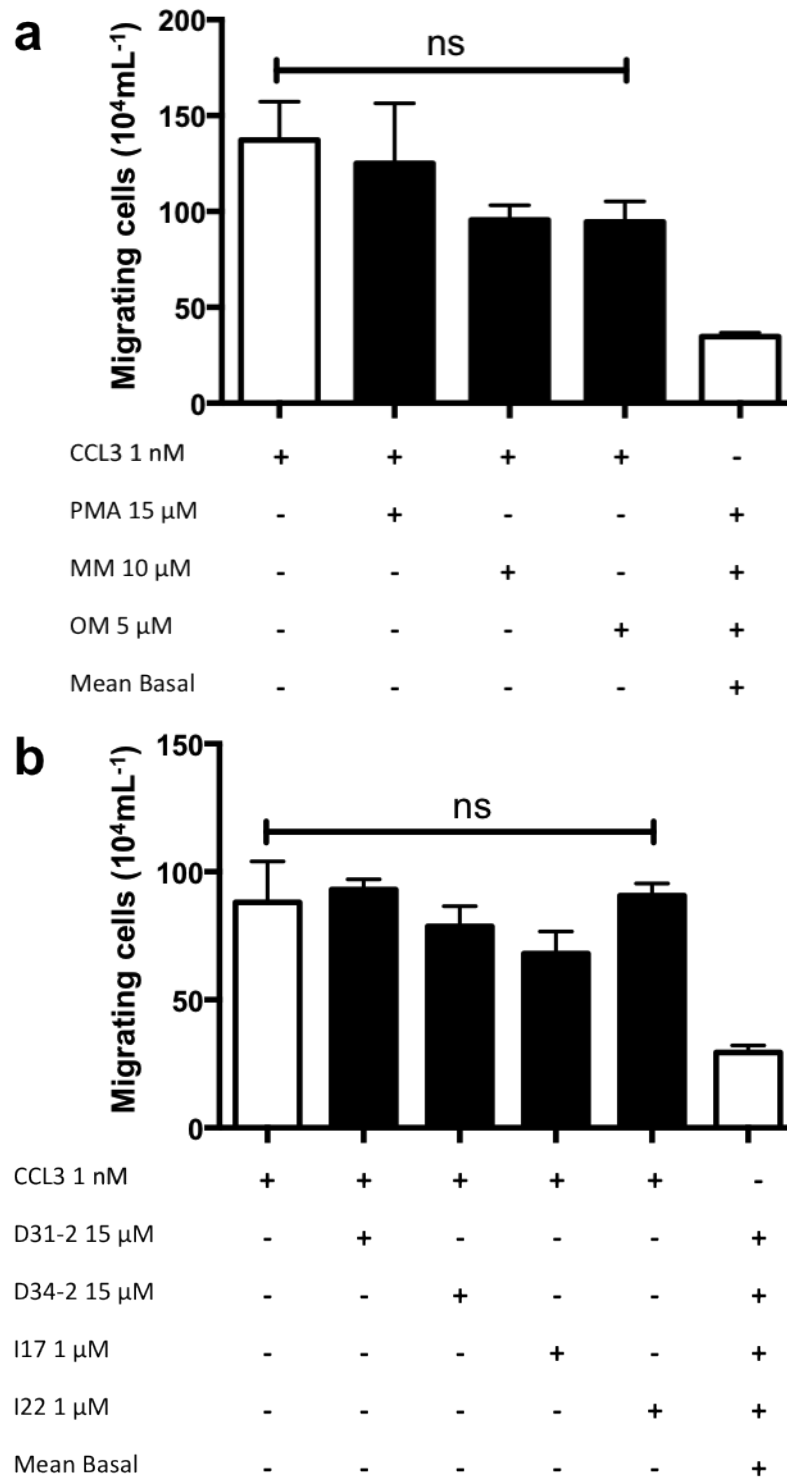


Figure 6.13: Other dynamin inhibitors have no significant effect on THP-1 cell chemotaxis. (a) MitMAB (MM) and OctMAB (OM) do not significantly affect THP-1 migration towards 1 nM. (b) Dynole 34-2 (D34-2) and Iminodyn 22 (I22) also have no significant effect. Results represent the mean \pm SEM of at least 3 independent experiments vs. vehicle control. (One-way ANOVA, Bonferroni multiple comparison. ns =not significant).

Pre-treatment of THP-1 cells with 10 μ M MiTMAB and 5 μ M OcTMAB resulted in no significant reduction in the ability of THP-1 cells to migrate towards a stimulus of 1 nM CCL3 compared to both the untreated vehicle control and cells treated with 15 μ M promystric acid (Figure 6.13a) ($p>0.05$, $n=6$). The non-competitive dynamin inhibitors dynole 34-2 and its inactive negative compound dynole 31-2 and iminodyn 22 and its inactive control compound iminodyn 17 were also used to treat THP-1 cells. These inhibitors exhibit higher GTPase IC_{50} values than dynasore. Pre-treatment of THP-1 cells with dynoles 34-2 and 31-2 and iminodyns 22 and 17 did not significantly affect THP-1 migration. Inhibitory compounds showed no significant reduction in THP-1 cell migration vs. their control compounds or the vehicle control. ($p>0.05$, $n\geq 3$). Although the migration was not significantly inhibited by MiTMAB, OcTMAB and the control compound iminodyn 17, a trend towards migration inhibition was visible in THP-1 cells. This could be a reflection of already published data by other groups that suggests MiTMAB and OcTMAB can reduce cancer proliferation [269]. These results were interesting because at the concentrations used for the experiments, the tested inhibitor should reduce the GTPase function of dynamin via distinct protein-inhibitor interactions. There are conflicting views of the importance of GTPase turn-over and the role of dynamin in migration [100, 149]. To examine whether the effect of dynasore on THP-1 migration was due to its inhibitory effect on GTPase turn-over, the dynasore analogue dyngo-4a, which has higher potency for dynamin 2 GTPase inhibition was used (Table 1) [142, 208].

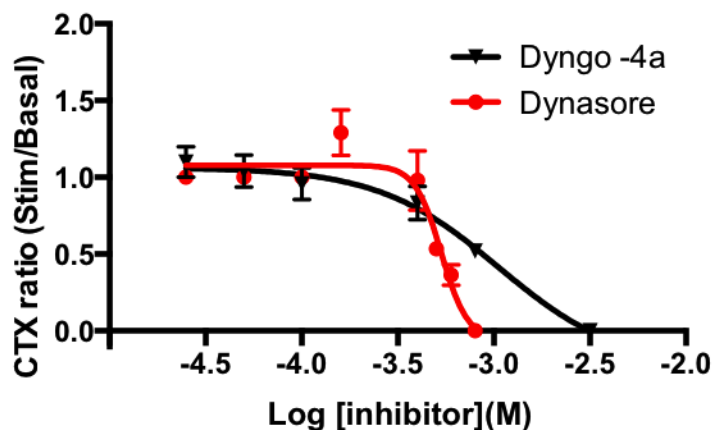


Figure 6.14: Dynasore is more potent inhibitor of CCR5 mediated THP-1 cell chemotaxis than its analogue dyngo-4a. Concentration response curves for inhibition of THP-1 migration towards 1 nM CCL3 in the presence of dynasore and its close structural analogue dyngo-4a. Results represent the mean \pm SEM of at least 3 independent experiments plotted as the ratio of stimulated over basal migration.

The effect of pre-treatment of THP-1 cells with increasing concentrations of dynasore and its analogue dyngo-4a, which shows a higher potency for GTPase inhibition, were used to plot concentration response curves for the inhibition of chemotaxis toward 1 nM CCL3 (Figure 6.14). Sigmoidal concentration response curves were fitted and IC_{50} values for dynasore (52 ± 4 nM) and dyngo-4a (103 ± 7 nM) were calculated. These data provide strong evidence that GTPase inhibition is not the most important factor for the effect of dynasore. The data also suggest that a structure-activity relationship can be formed based around alterations in the dynasore terminal aryl-ring hydroxyl moieties. To exemplify these functional differences and to determine if the other dynamin inhibitors have any effect on migration at higher molarity, an equimolar comparison of the inhibitors on THP-1 migration was performed.

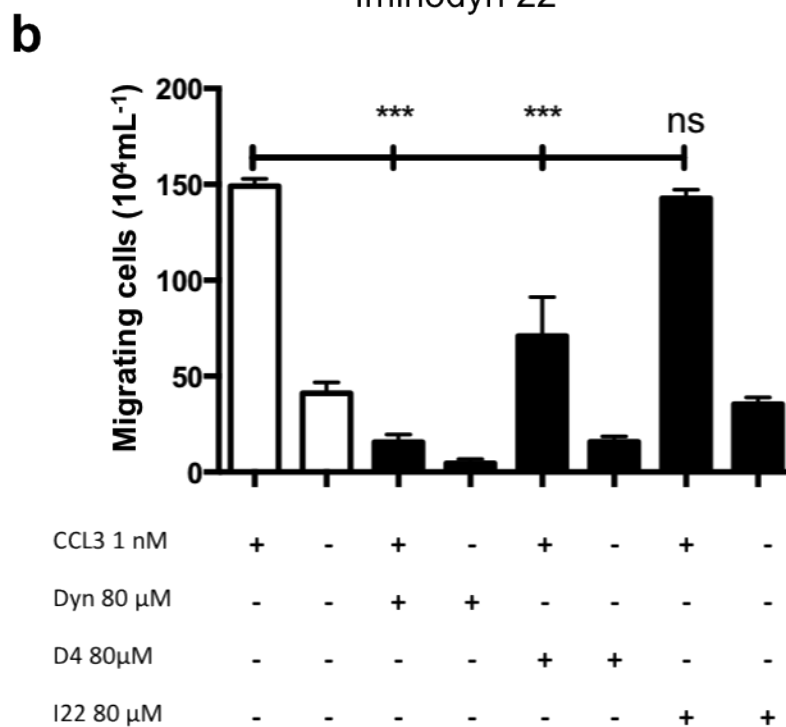
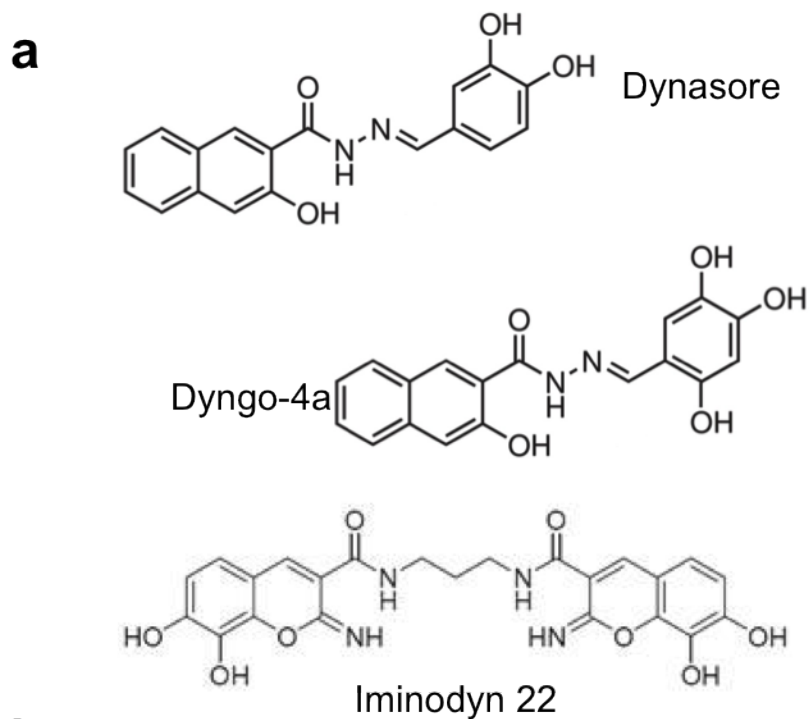


Figure 6.15: Equimolar comparison of structurally homologous dynamin inhibitors. (a) Chemical structures of most similar dynamin inhibitors. (b) Inhibition of THP-1 migration towards 1 nM CCL3 reveals structure activity relationship (One-way ANOVA, Bonferroni multiple comparison. *** indicates $p \leq 0.001$ vs. vehicle control. Results represent the mean \pm SEM of at least 3 independent experiments).

Being analogues, dynasore and dyngo-4a share a high level of structural similarity however, the iminodyn 22 inhibitor also has obvious structural homology and has also been shown to have a significantly higher GTPase inhibition IC_{50} [206] (Figure 6.15a). When used at 80 μ M clear comparisons can be made about the efficacy of these dynamin inhibitors to block migration and how this efficacy has no bearing on their GTPase IC_{50} values (Figure 6.15b). Based on these three compounds it can be concluded that the inhibitory effects of a compound on migration are inversely proportional to their GTPase inhibitory potency. The remaining inhibitors were also used at 80 μ M to determine if this correlation was maintained.

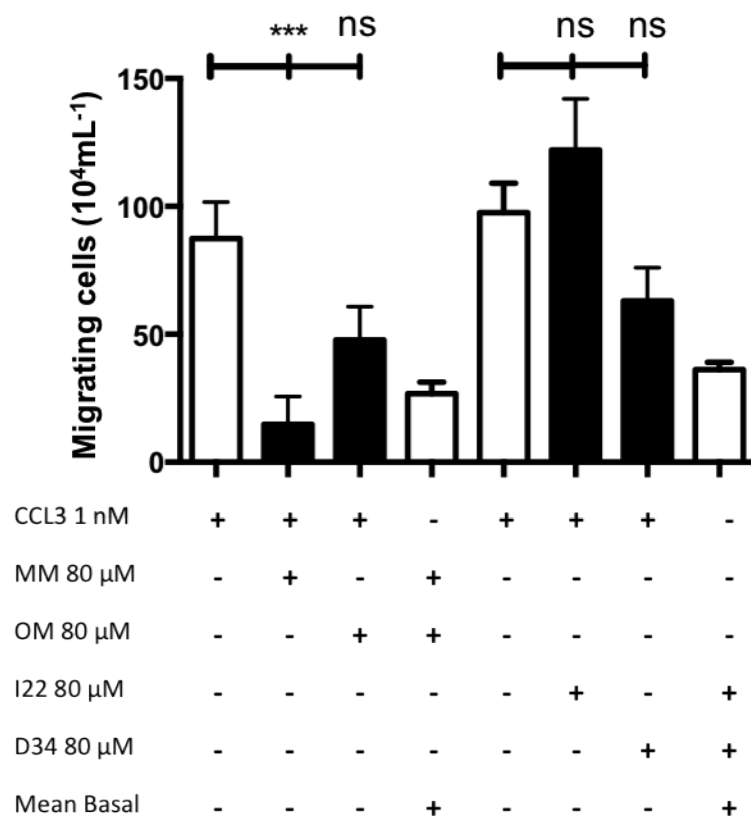


Figure 6.16: Equipolar comparison of MiTMAB, OctTMAB and Dynole 34-2. Only cells treated with 80 μ M MiTMAB show a significant reduction in THP-1 migration towards (p \leq 0.001 n \geq 3). Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. * indicates p \leq 0.001 vs. vehicle control. ns = not significant).**

Of the remaining dynamin inhibitors, only MiTMAB was able to significantly reduce THP-1 cell migration toward CCL3 at 80 μ M ($p \leq 0.001$, $n=3$), with OcTMAB and dynole 34-2 showing a non-significant trend towards inhibition ($p > 0.05$, $n \geq 3$) (Figure 6.16). Before conclusions can be drawn about these observations it is important to determine if the 80 μ M treatments used are showing any cytotoxic effects.

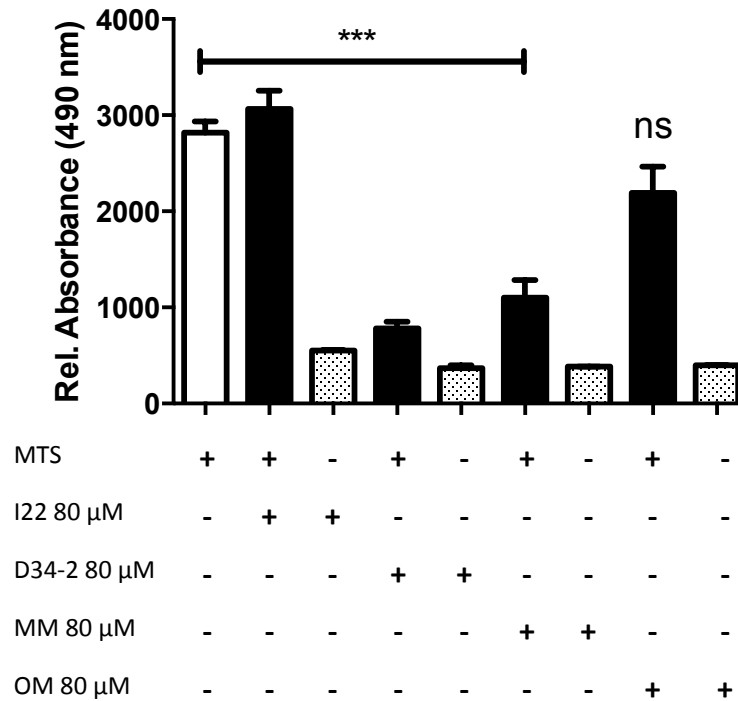


Figure 6.17: MiTMAB and Dynole 34-2 show significant cytotoxicity at 80 μ M. THP-1 cells treated with 80 μ M dynamin inhibitors for 0.5hr before incubation with MTS for 5hr reveal significant reduction in viability associated with MM and D34-2 ($p \leq 0.001$ $n \geq 3$). Results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison.* indicates $p \leq 0.001$ vs. vehicle control. ns = not significant).**

MTS cytotoxicity assessment of THP-1 cells treated with 80 μ M iminodanil 22, dynole 34-2, MiTMAB and OcTMAB revealed that dynole 34-2 and MiTMAB significantly reduced THP-1 cell viability after 5hr treatment (Figure 6.17) ($p \leq 0.001$, $n=3$). Iminodanil 22 and OcTMAB showed no significant reduction in viability although a trend was observed towards reduced viability with OcTMAB treatment ($p > 0.05$, $n=3$). Considering MiTMAB and OcTMAB are analogues of one another this reduction in viability seen with OcTMAB treatment is likely to represent cytotoxic effects and is sufficient to explain the non-significant trend in migration observed with 80 μ M treatment of THP-1. Taken as a whole these data suggest that any reduction in migration seen with higher concentrations of dynamin inhibitors other than dynasore and dyngo-4a is due to cytotoxicity and that the anti-migratory effects of these inhibitors is not due to dynamin 2 GTPase inhibition as their potency does not correlate with their GTPase IC_{50} .

To validate the observations seen in THP-1 cells, the dynamin inhibitors dynasore and dyngo-4a were used to treat HeLa.RC49 cells in the wound-healing assay. Confluent HeLa.RC49 cells in a low serum environment show an ability to migrate into a scratch, which allows an assessment of the effect of inhibitors on migration. HeLa.RC49 cells show an un-stimulated basal regrowth that is increased by stimulation of CCR5 and is blocked by 80 μ M dynasore pre-treatment. Figure 6.18 shows representative images for these assays and shows how quantification of regrowth is determined.

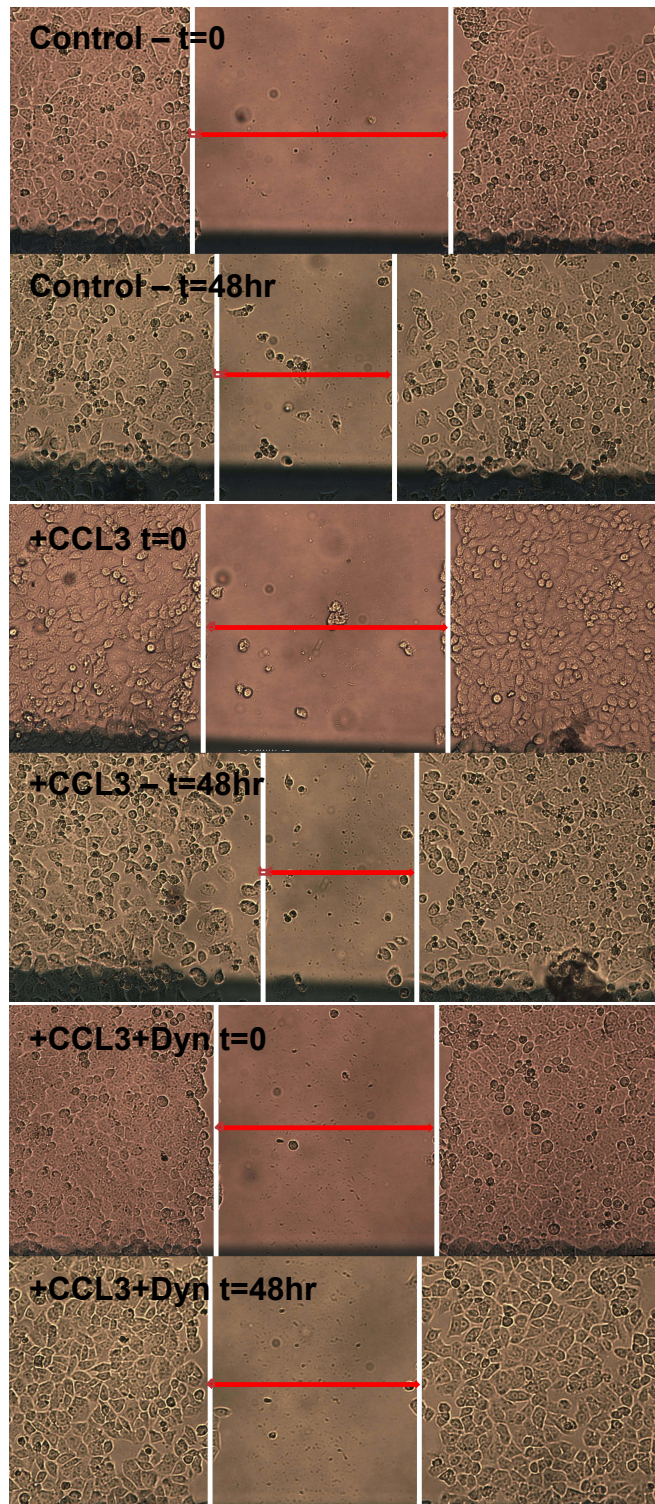
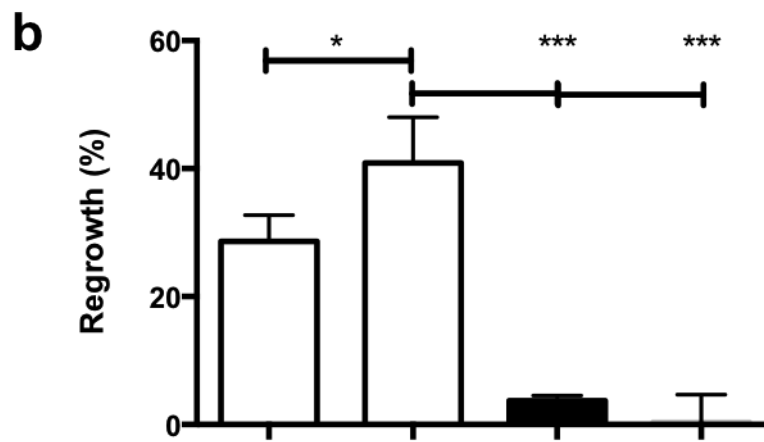
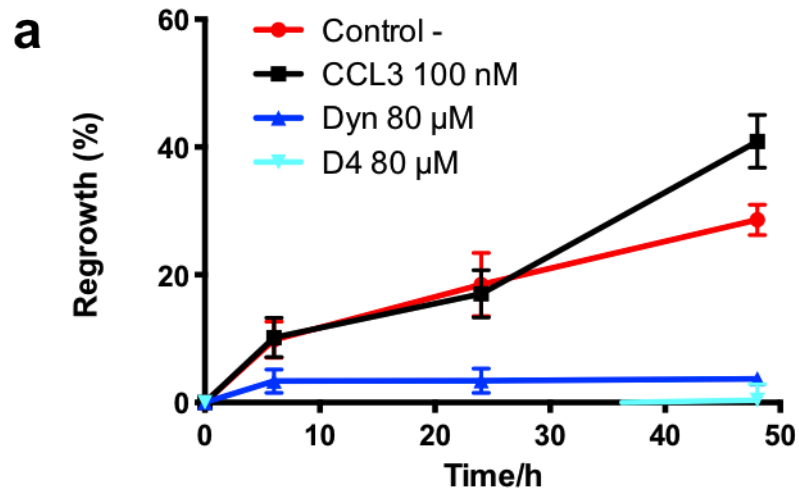


Figure 6.18: The scratch or 'wound healing' assay reveals the effect of dynasore on HeLa.RC49 migration. Scratch assay performed on confluent HeLa.RC49 monolayer demonstrates the differences between basal and CCL3 stimulated healing and that for cells treated with 80 μ M dynasore for 0.5hr before stimulation.

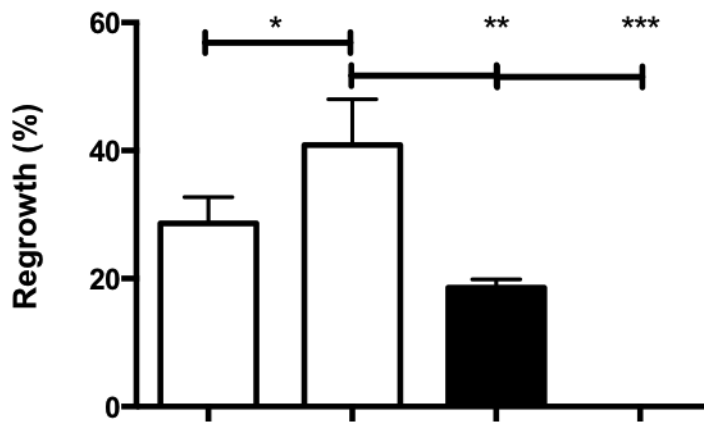
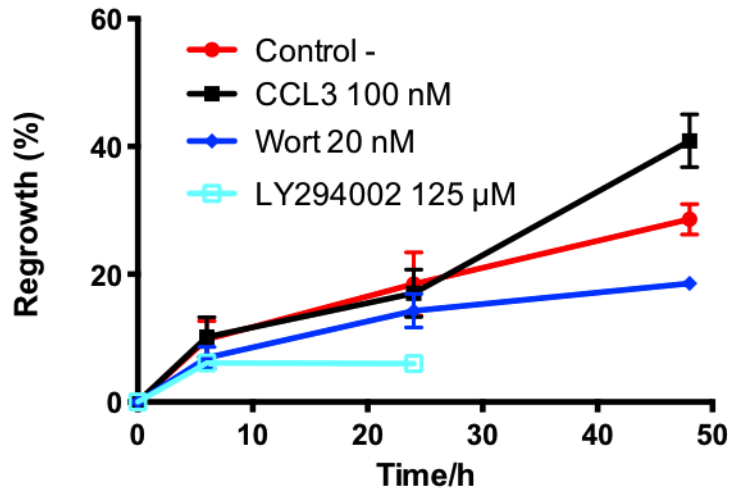


CCL3 100 nM	-	+	+	+
Dyn 80 μM	-	-	+	-
D4 80 μM	-	-	-	+

Figure 6.19: Quantification of HeLa.RC49 wound healing assay in the presence of dynasore and dyngo-4a. (a) A graphical representation of wound healing in HeLa.RC49 treated with dynasore (Dyn) and dyngo-4a (D4) in comparison to basal and stimulated controls (quantification method described in materials and methods). **(b)** dynasore and dyngo-4a significantly reduce wound healing in HeLa.RC49 vs. CCL3 stimulated control. Results represent the mean \pm SEM of 3 independent experiments run in duplicate (One-way ANOVA, Bonferroni multiple comparison. *** indicates $p \leq 0.001$ vs. CCL3 stimulated vehicle control).

Both dynasore and dyngo-4a are able to significantly reduce CCL3 stimulated HeLa.RC49 wound healing (Figure 6.19) ($p < 0.001$, $n=3$ vs. stimulated control). Wound healing is decreased to 2% compared to 40% for simulated controls. Dyngo-4a appears to be more efficacious in this assay, although this observation is not significant. These data clearly support the idea that dynamin is required for fibroblast migration [158]. There is, however, some evidence which supports the idea that the mechanisms of migration in large adherent cells and monocytes differ regarding the polarisation via PI3K function. To test if PI3K is required for HeLa.RC49 migration, the inhibitors LY294002 and wortmannin were used to pre-treat cells before a scratch healing assay.

Both LY294002 and wortmannin pre-treatment of HeLa.RC49 cells results in a significant reduction in wound healing vs. the stimulated controls ($p < 0.001$, $n=3$) (Figure 6.20). Cells treated with LY294002 completely detach from the mounting medium after 48hr, which indicates that the cells are either dying or are unable to remain adherent due the effect of PI3K inhibition. This can be taken as evidence that HeLa.RC49 fibroblasts are more sensitive to PI3K inhibition than monocytic THP-1 cells, which in turn would support the observations of Volpe *et al.* [115].



CCL3 100 nM	-	+	+	+
Wort 20 nM	-	-	+	-
LY294002 125 μM	-	-	-	+

Figure 6.20: PI3K inhibitors reduce CCL3 stimulated wound healing in HeLa.RC49 cells. A graphical representation of wound healing in HeLa.RC49 treated with LY294002 and wortmannin (Wort) in comparison to basal and stimulated controls. (b) LY294002 and Wortmannin significantly reduce wound healing in HeLa.RC49 vs. CCL3 stimulated control. Results represent the mean \pm SEM of 3 independent experiments run in duplicate (One-way ANOVA, Bonferroni multiple comparison.*** indicates $p \leq 0.001$ vs. CCL3 stimulated vehicle control).

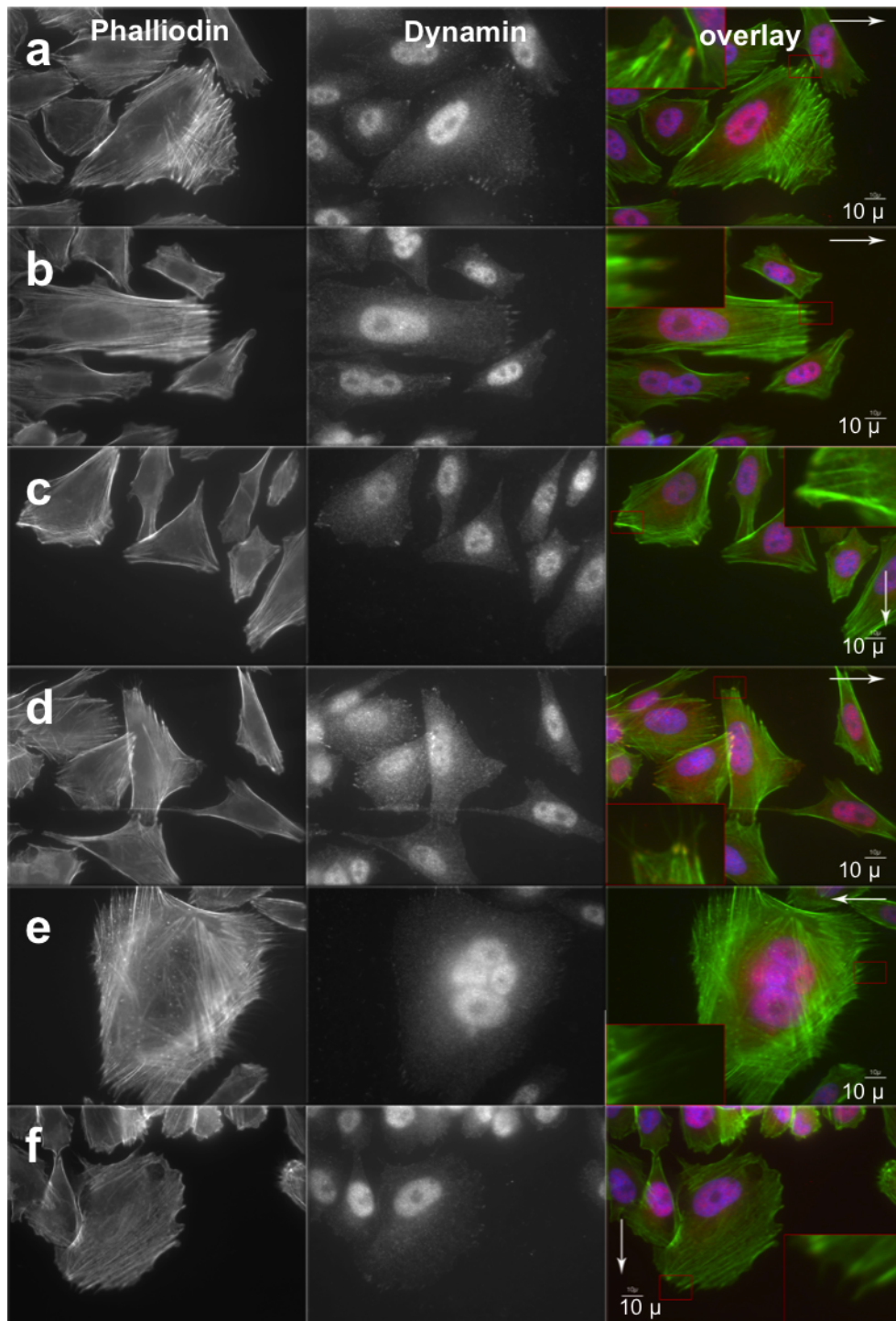


Figure 6.21: Dynasore does not affect dynamin localisation but does alter membrane structure and the ability for CHO.CCR5 cells to polarise. Fluorescence microscopy images of Alexa-488 phalloidin (Phal) anti-dynamin Ab stain (Dyn) and overlay + DAPI (o/l). (a) Negative control. (b) + 2hr 100nM CCL3 stimulation. (c) 0.5hr dynasore 80 μ M (d) + 2hr 100 nM CCL3 stimulation. (e) 24hr dynasore 80 μ M (f) + 2hr 100 nM CCL3 stimulation. Acquired with Zeiss axiovision 2 system. \rightarrow = direction of scratch.

The effect of dynasore on dynamin localisation in CHO.CCR5 cells was assessed in order to try and determine abnormal dynamin localisation might explain the observed reduction in migration. CHO.CCR5 monolayers were cultured and scratched prior to treatment with 80 μ M dynasore for 0.5hr or 24hrs and stimulation with 100 nM CCL3 for 2hr. The scratches were introduced to allow observations regarding the ability of cells to polarise to be made. Dynamin localisation was observed in both unstimulated and stimulated controls around the nucleus and at the tips of actin stress fibers (Figure 6.21a&b) at locations thought to relate to base filopodia or focal adhesions. Controls cells show a clear polarisation towards scratches that is more pronounced in CCL3 stimulated cells. Cells treated with dynasore for 0.5hr prior to stimulation show similar levels of polarisation as control unstimulated cells which could be attributed to the basal polarisation during 24h incubation period, but these cells also show dynamin localisation at the nucleus and at actin fibre termini (Figure 6.21c&d). Cells treated for the 24hr incubation with 80 μ M dynasore showed a distinct lack of polarisation although once again dynamin localisation was not altered noticeably. These cells also seemed to exhibit a higher number of membrane protrusions, reminiscent of filopodia, which may suggest that whilst dynasore does not prevent dynamin localisation but prevent cells polarising under basal conditions or in stimulation, by CCL3. This may have explained the increase in filopodia which would normally be associated with the leading edge of cells; where this leading edge could not be 'identified' by the cell the filopodia appear uniformly.

6.3.4. Dynamin inhibitors reduce CCL3 stimulated calcium release through non specific mobilisation of calcium stores

The evidence provided in chapter 4 and in the literature [75] supports the idea that calcium release and migration are independent phenomena downstream of CCR5, this does not explain fully the effects of dynasore. To determine if the observed reduction in calcium release is a result of dynamin inhibition all available dynamin inhibitors were used to treat THP-1 cells prior to calcium flux assays.

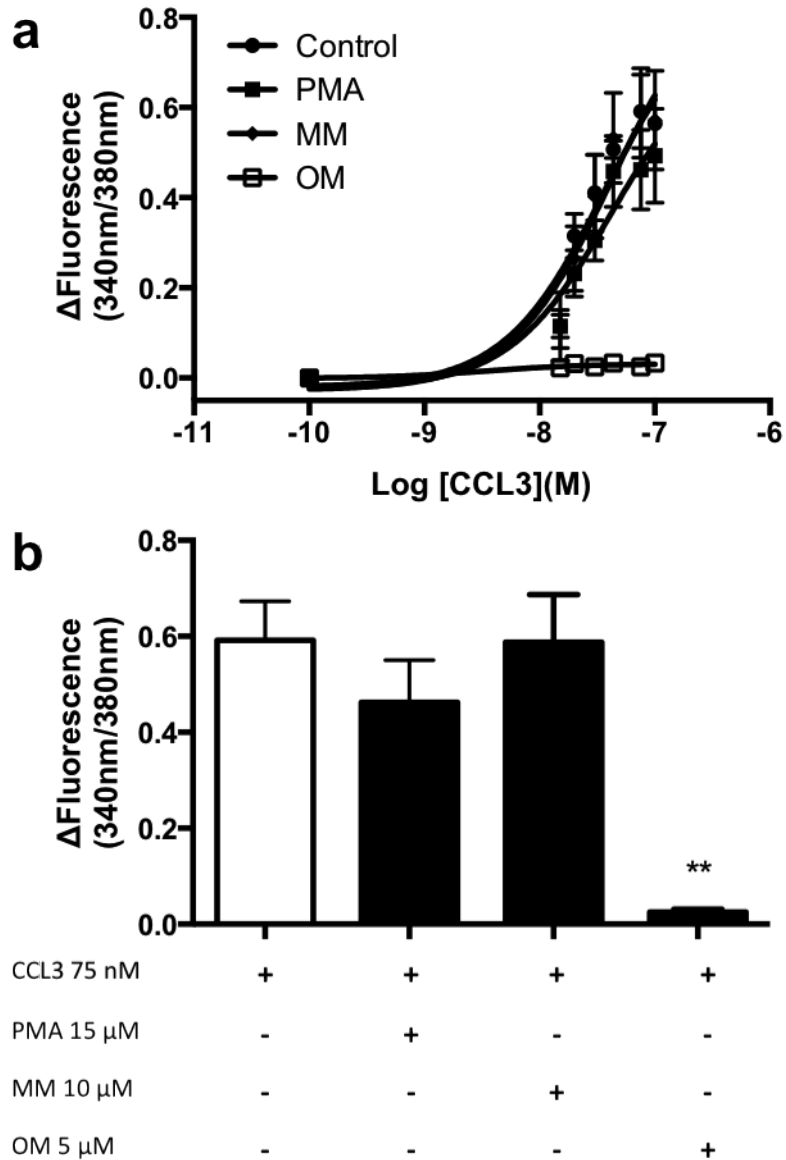


Figure 6.22: OcTMAB inhibits CCL3 stimulated intracellular calcium release in THP-1 cells. (a) Concentration response for CCL3 stimulation of THP-1 cells with and without 0.5hr pre-treatment with 10 μM MiTMAB (MM), 5 μM OcTMAB (OM) and negative control compound promystric acid (PMA). (b) OM significantly reduces calcium release stimulated by 75 nM CCL3 ($p \leq 0.01$ $n \leq 3$). Results represent the mean \pm SEM of 3 independent experiments run in duplicate (One-way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.001$ vs. vehicle control).**

Pre-treatment of THP-1 cells with MiTMAB, OcTMAB and control compound promystric acid for 0.5hr prior to calcium flux assay revealed that OcTMAB treatment significantly reduces calcium release (Figure 6.22.). Predicted efficacy and EC₅₀ values for promystric acid and MiTMAB treated cells are not significantly different than the untreated control ($p>0.05$, $n=4$). OcTMAB treatment results in a significant reduction in calcium release stimulated by 75 nM CCL3 ($p\leq 0.001$, $n=4$ vs. negative control), promystric acid and MiTMAB showed no significant effect ($p>0.05$, $n=4$).

The predicted maximal calcium release (stimulation over basal) for dynole 31-2 treated cells was 0.847 ± 0.156 . A sigmoidal fit was not possible for dynole 34-2 (assuming hill co-efficient of 1), so efficacy predictions could not be made. CCL3 stimulated calcium release at 75 nM CCL3 was however significantly lower vs. dynole 31-2 treated cells ($p\leq 0.001$, $n=4$). CCL3 stimulated calcium release in iminodyn 17 treated THP-1 cells showed an efficacy of 0.723 ± 0.118 vs. 0.762 ± 0.184 for iminodyn 22 treated cells. The predicted maximal CCL3 efficacy for dynole 31-2 and iminodins 17 and 22 were not significantly different from one another ($p>0.05$, $n=4$, one-way ANOVA with Bonferroni multiple comparison) and calcium release at 75 nm CCL3 was not significantly different with these treatments (Figure 6.23b) ($n>0.05$, $n=4$).

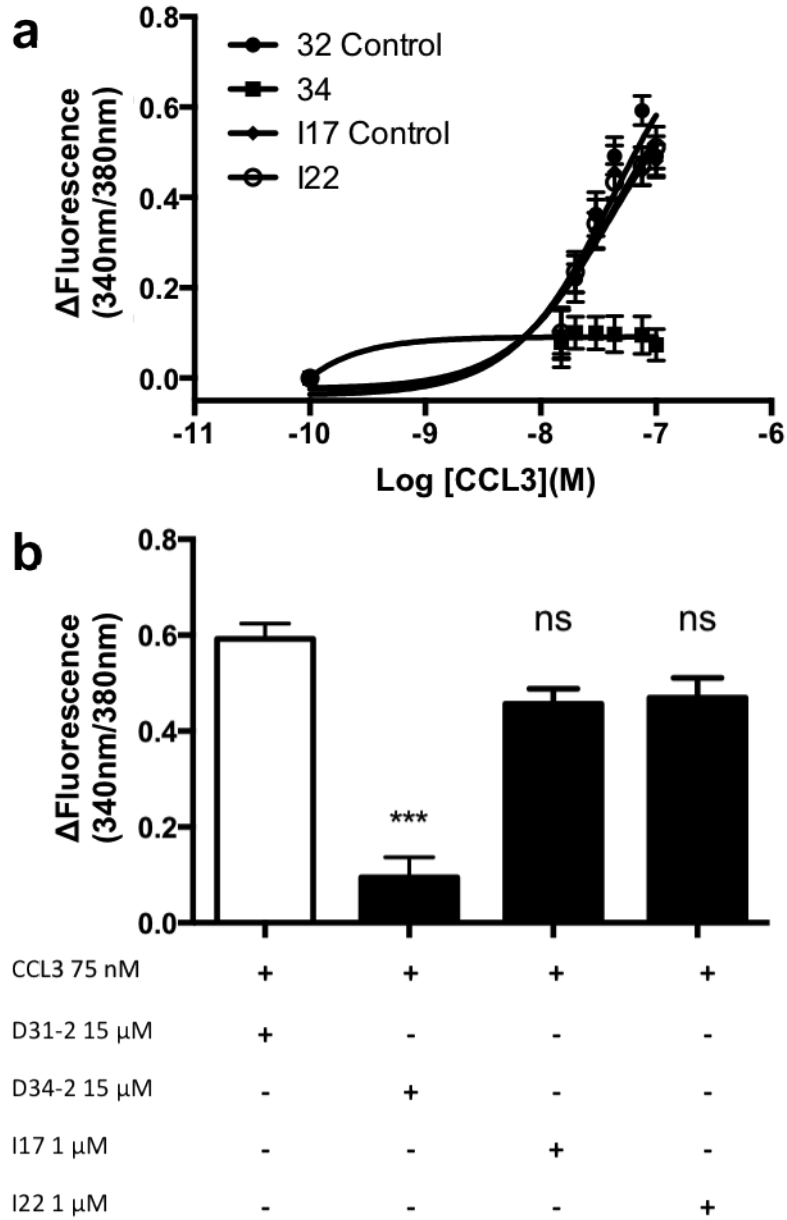


Figure 6.23: Dynole 34-2 (D34-2) inhibits CCL3 stimulated intracellular calcium release in THP-1 cells. (a) Concentration response for stimulation of THP-1 with increasing concentrations of CCL3 with and without 0.5hr pre-treatment with 15 μM D34-2 and its negative control dynole 31-2 (D31-2) and 1 μM iminodyn 22 (I22) and its negative control iminodyn 17 (I17). (b) D34-2 significantly reduced calcium release stimulated by 75 nM CCL3 ($p \leq 0.001$ $n \leq 3$) Results represent the mean \pm SEM of 3 independent experiments run in duplicate (One-way ANOVA, Bonferroni multiple comparison. *** indicates $p \leq 0.001$ vs. vehicle control).

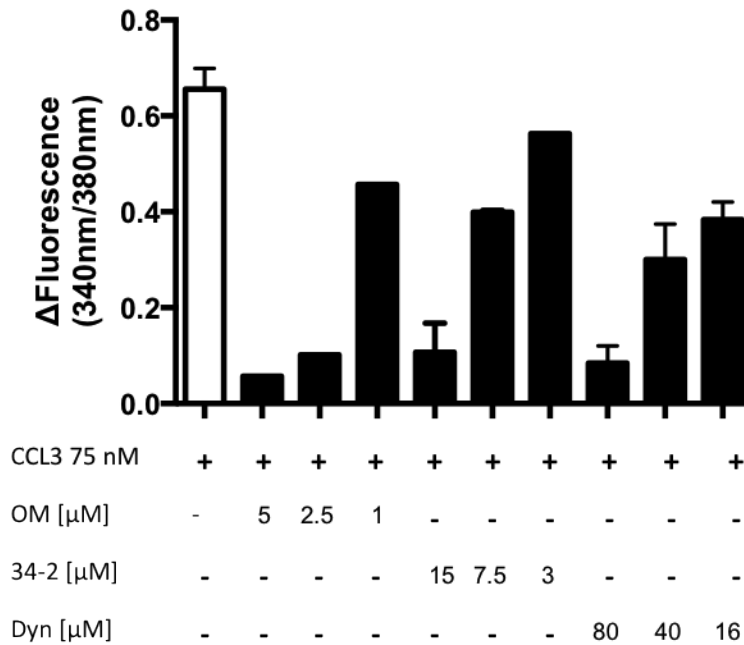


Figure 6.24: Dynamin inhibitors that block calcium release do so in a concentration dependent fashion. A reduction in inhibitor concentration by 1/2 and 1/5 allows recovery of THP-1 calcium release stimulated by 75 nM CCL3. Results represent the mean \pm SEM of at least 2 independent experiments.

Dynasore (80 μ M) OcMAB (5 μ M) and Dynole 34-2 (15 μ M) significantly affect calcium release, however, the means by which this occurs does not seem to be based on their known potency to block the GTPase activity of dynamin. A correlation of inhibitor effects with dynamin binding sites is also not detectable; the observed effect of OcTMAB is absent in the analogue MiTMAB. To determine if the effects of these inhibitors are concentration dependent, calcium release was assayed in the presence of the original concentration then cells were treated with decreasing concentrations of inhibitor. THP-1 cells (Figure 6.24) demonstrate that the effects of dynasore, OcTMAB and dynole 34-2 are concentration dependent suggesting the molecules are acting as inhibitors of a process on which calcium release is dependent. Dynasore has been shown to be non-cytotoxic at treatment concentrations, but before the effects of OcTMAB and dynole 34-2 can be explained it is important to show the results are not the result of concentration dependent cell killing.

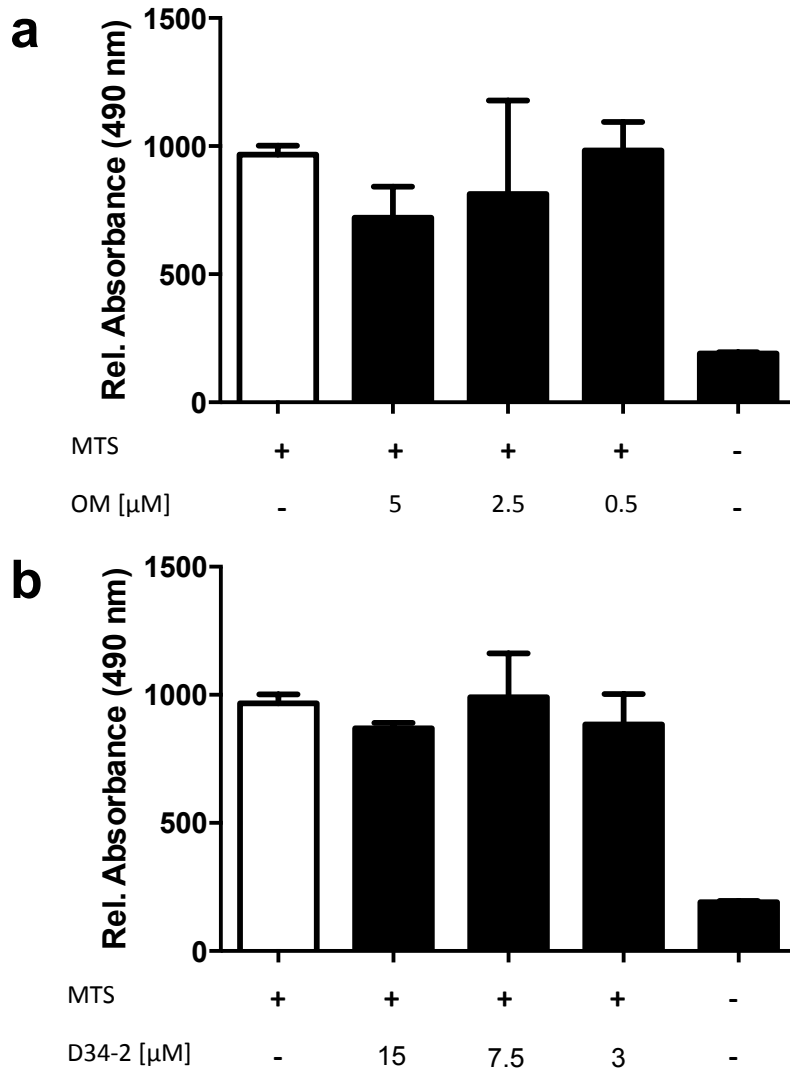


Figure 6.25: OcTMAB and dynole 34-2 do not show cytotoxicity at concentrations that inhibit calcium release in THP-1 cells. (a) There is no significant reduction in viability in THP-1 treated with OcTMAB for 0.5hr then incubated with MTS for 5hr (n=3). (b) Likewise dynole 34-2 (D34-2) treatment shows no significant reduction in viability. Results represent the mean \pm SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison).

MTS cytotoxicity assay was used to determine if the observed decrease in calcium release seen in THP-1 treated with OcTMAB and dynole 34-2 could be attributed to concentration dependent cell death (Figure 6.25). THP-1 cells treated with 5, 2.5 and 1 μ M OcTMAB showed no significant reduction in cell viability after 5 hours compared with untreated cells (Figure 6.25a)($p>0.05$, $n=3$). Likewise, THP-1 cells treated with decreasing concentrations of dynole 34-2 used in

calcium flux assay also showed no significant reduction in cell viability ($p > 0.05$, $n = 3$ vs. vehicle control). These data demonstrate that the reduction in calcium release seen with the dynamin inhibitors is not based on cytotoxic effects which presents an interesting conundrum as to why other dynamin inhibitors show an ability to alter calcium release and, where dynasore is concerned, represent a potential link between chemotaxis and calcium release which are considered to be independent in THP-1 cells. Before the mechanisms of the effect on calcium release of these drugs can be investigated further it was important to determine if the observations can be a result of non-specific calcium releases induced by the inhibitors. Nonspecific calcium release occurs when compounds act directly on calcium stores bypassing receptor mediated signalling. Thapsigargin is a widely used 'non-specific' stimulator of calcium release (although its mode of action is specific) and it can be used to determine the effect of other drugs on calcium stores [93, 270].

THP-1 cells were pre-treated for 0.5hr with decreasing concentrations of dynasore, OcTMAB and D-34 (as used previously) prior to calcium flux assay stimulated by $1 \mu\text{M}$ thapsigargin (Figure 6.26a). The rationale behind the experiment is that should any of the dynamin inhibitors directly affect calcium stores than the amount of calcium released by thapsigargin stimulation would be reduced compared to control cells. Because receptor activation is not taking place in this assay, any effects could then be categorised as 'non-specific' calcium release. All treatment concentrations of dynasore and OcTMAB and all but the lowest treatment concentration of D-34 resulted in a significant reduction in thapsigargin stimulated calcium release ($p \leq 0.001$, $n = 3$). The reductions in thapsigargin stimulated calcium release also followed a concentration dependent pattern further validating the idea that 'non-specific' calcium release is occurring through dynamin inhibitor treatment. The implications of this observation are far reaching both with regard to the actual mechanism and to relevance to other cell lines where dynasore is often used in calcium release based experimentation [201].

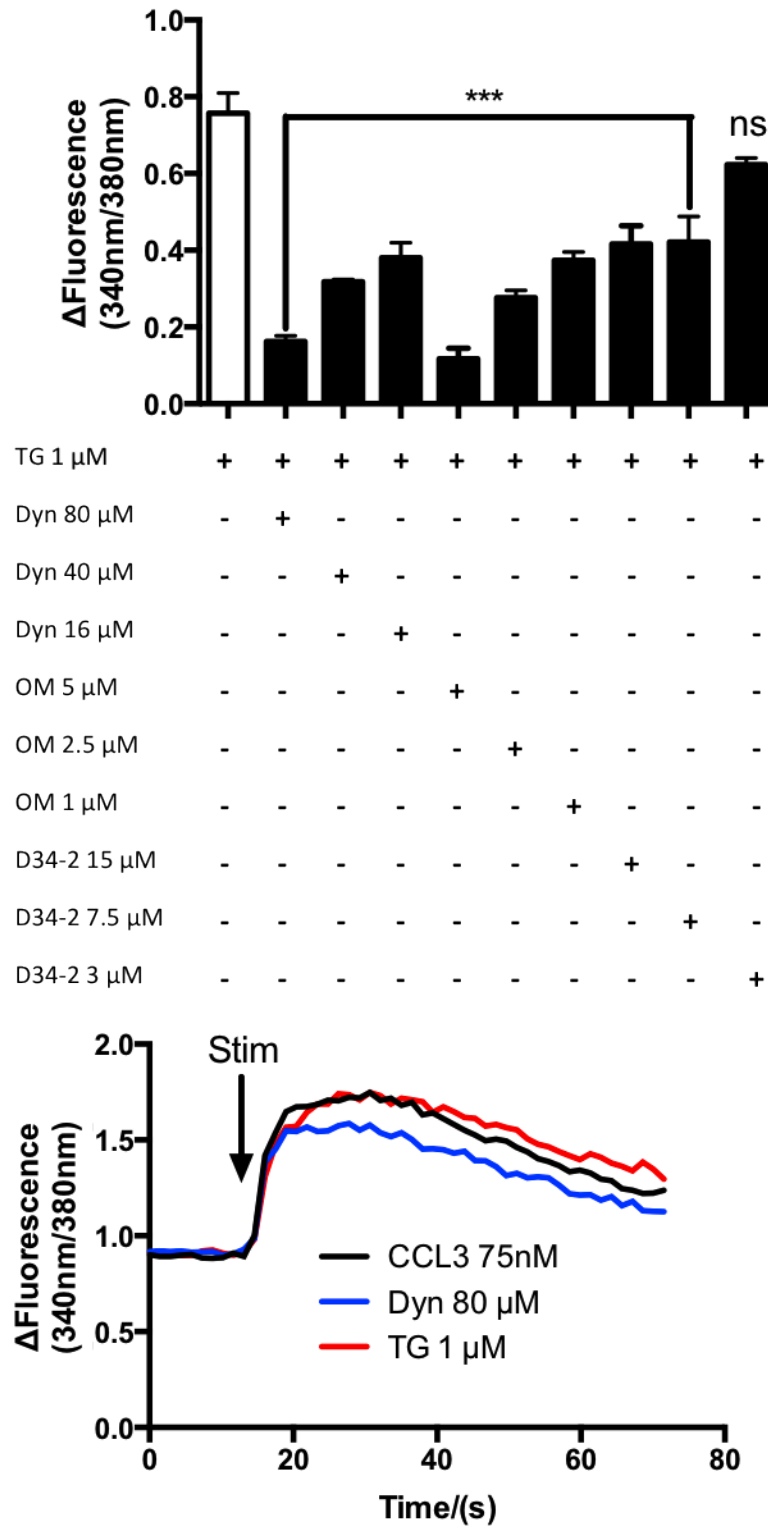


Figure 6.26: Dynasore reduces thapsigargin stimulated calcium release in THP-1 cells. (a) Pre-treatment of THP-1 with dynasore (Dyn), OctTMAB (OM) and dynole 34-2 (D34-2) significantly reduces thapsigargin (TG 1 μM) stimulated calcium release in a concentration dependent fashion ($p \leq 0.001$ $n \geq 3$). (b) Single traces of calcium mobilisation over time. All results represent

the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison.*** indicates $p \leq 0.001$ vs. control).

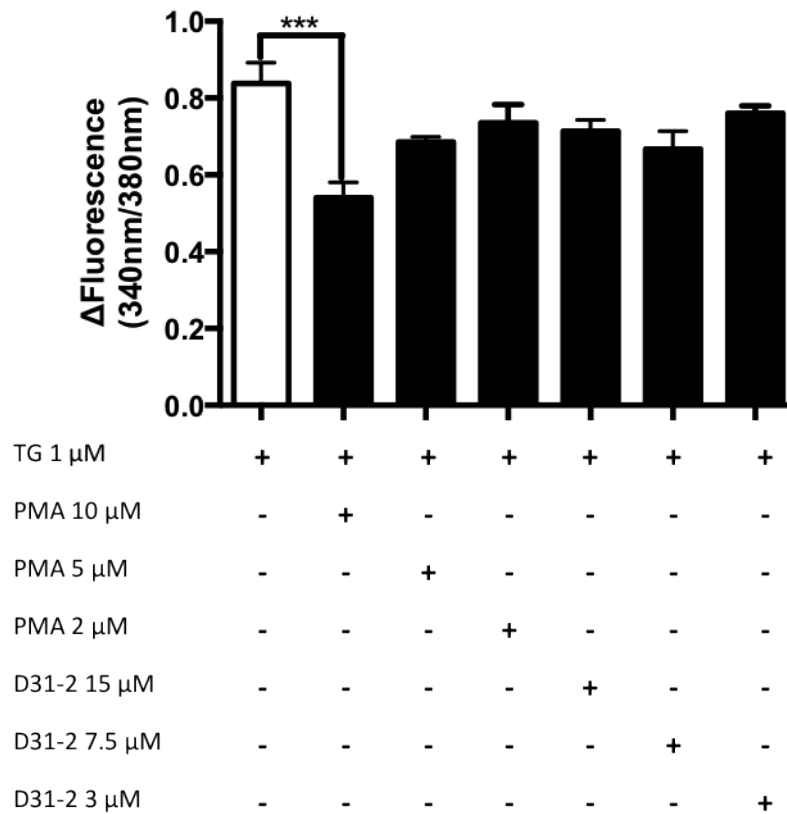


Figure 6.27: Dynamin inhibitor negative controls with the exception of promystric acid have no significant effect on TG induced calcium release. (a) Promystric acid (PMA) at higher concentrations significantly reduces calcium release in THP-1 stimulated by 75 nM CCL3, lower concentrations and dynole 31-2 have no significant effect. All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. *** indicates $p \leq 0.001$ vs. control).

The negative control compounds for the dynamin inhibitors that had been shown to reduce calcium flux were also used to treat THP-1 cells prior to thapsigargin stimulated calcium release (Figure 6.27.). These compounds validated the model and demonstrated that all treatment concentrations (with the exception of 10 μ M promystric acid) had no significant effect on calcium release ($p > 0.05$, $n = 3$). The significant ($p \leq 0.001$, $n = 3$) but partial reduction in thapsigargin stimulated calcium release shown by 10 μ M promystric acid treatment is less easy to explain. Promystric acid did show a non-significant trend toward reducing calcium release in THP-1 cells, which might indicate the observed effects of OcTMAB are a result of structural effects of the homology series which do not affect dynamin GTPase function.

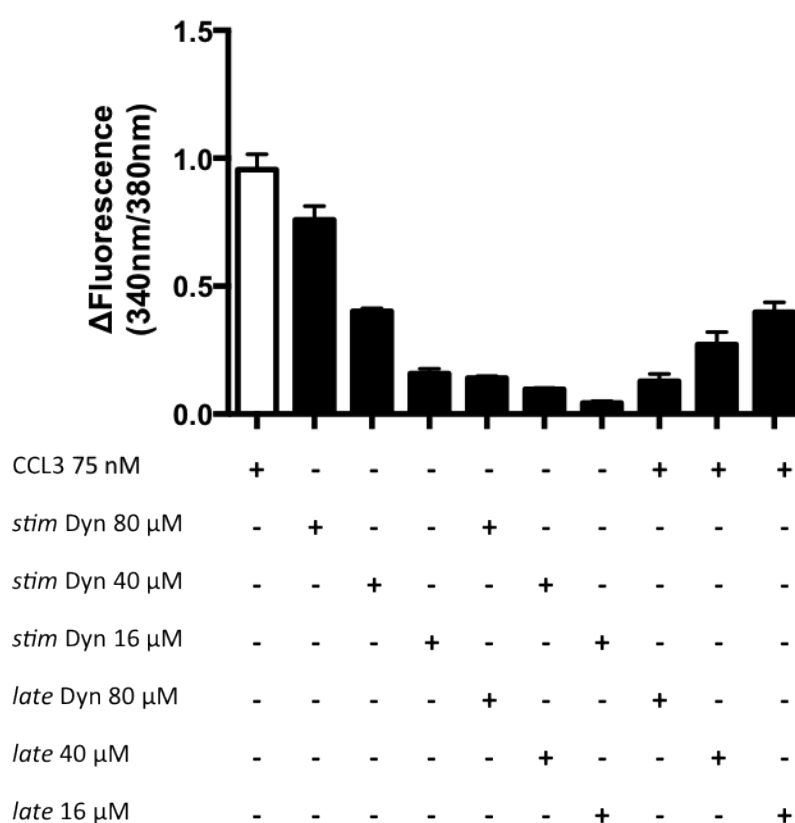


Figure 6.28: Dynasore stimulates calcium release in THP-1 cells. Stimulation with dynasore (*stim Dyn*) causes intracellular calcium release in THP-1 in a concentration dependent fashion. Late inhibition of THP-1 cells, 1 minute prior to stimulation (*late Dyn*), blocks stimulatory

effects of Dyn and 75 nM CCL3. All results represent the mean \pm SEM of at least 3 independent experiments.

Finally, dynasore was used to stimulate untreated THP-1 cells to determine if the compound could stimulate calcium release. Untreated THP-1 cells were prepared for calcium release assay as usual and then stimulated with 16, 40 and 80 μ M dynasore or 75 nM CCL3 as a positive control (Figure 6.28). Stimulation with 80 μ M dynasore resulted in a stimulated calcium release not significantly different from 75 nM CCL3 and the concentration dependent nature of the stimulation was maintained.

6.3.5. Grb2 plays a functional role in CCR5 mediated chemotaxis in THP-1 cells

As discussed in the introduction, modulation of dynamin by protein interactions at the PRD can not only increase GTPase function of dynamin, but also its ability to polymerise and interact with actin fibres. This interaction has been shown to promote the removal of gelosin caps from actin barbed ends which promotes polymerisation and podosome formation [164]. SH3 domain containing protein interactions with dynamin via its PRD enhance the ability of dynamin to polymerise and also complexes the protein with crucial signal transduction mediators such as GTP exchange factors and non-receptor tyrosine kinases [100, 135, 159]. Grb2 interaction with the dynamin 2 PRD domain is well documented [158, 165, 271] and knock down and over expression of the protein has been shown to effect migration [135]. Grb2 represented an interesting target for investigation of CCR5 mediated migration as there is some evidence that CCR5 can interact with the protein [101, 196].

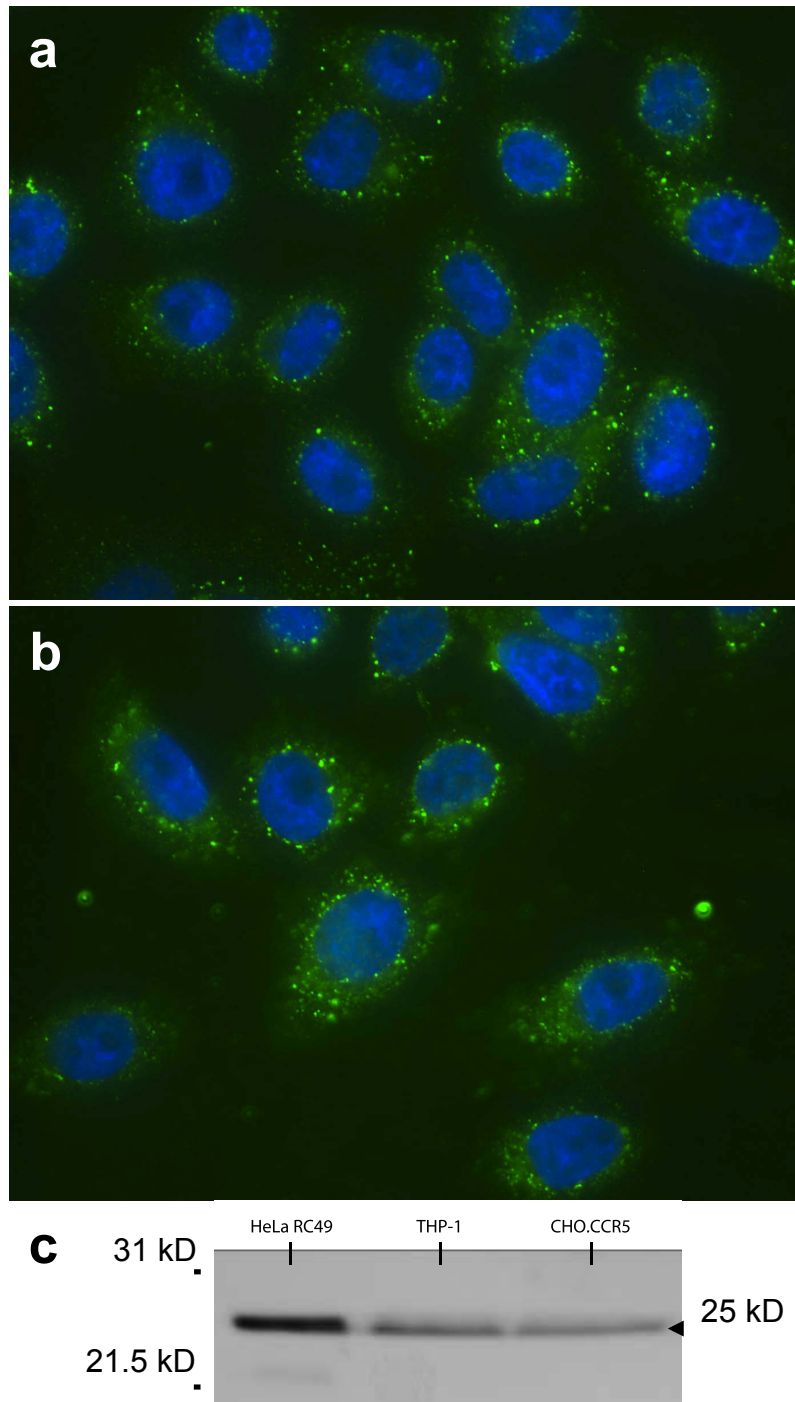


Figure 6.29. Grb2 is present in THP-1, HeLa.RC49 and CHO.CCR5 cells with cytosolic distribution. (a) Anti-Grb2 stain with Alexa-488 tagged secondary, in unstimulated HeLa.RC49. (b) In the presence of 100 nM CCL3 (DAPI nuclear stain). (c) Western blotting reveals Grb2 is present in HeLa.RC49, THP-1 and CHO.CCR5. Images representative of population, acquired with Zeiss axiovision 2 system.

An anti-Grb2 monoclonal antibody was used to determine if Grb2 is present in the model cell lines. Initially HeLa.RC49 cells treated with 100 nM for CCL3 for 1hr or left untreated were fixed and stained with anti-Grb2 Ab and FITC tagged anti mouse Ab. Grb2 is localised throughout the cytoplasm, but not in the nucleus. In CCL3 treated cells a slight increase in Grb2 around the ER/Golgi was observed, but this was not quantified (Figure 6.29b). Western blotting was used to identify Grb2 expression in CHO.CCR5, HeLa.RC49 and THP-1 cells (Figure 6.29c). The Grb2 SH2 domain inhibitor CGP78850 is a rationally designed molecule and has been shown to inhibit RTK mediated chemotactic signalling. RTK chemotaxis relies on the Grb2-RAS-SOS-PI3K axis [112, 113] which is distinct from signal transduction via chemokine receptors. The effect of Grb2 inhibition on chemokine receptor mediated migration has not been explored yet, so the effect of CGP78850 on THP-1 migration was investigated. The IC_{50} of CGP78850 for isolated Grb2 is in the low μ M range, however, due to its hydrophilic nature cell permeability is poor with cytoplasmic concentrations approximately 50 fold lower than those outside the cell [113]. For this reason the treatment concentrations used were significantly higher than might be expected based solely on published potency values.

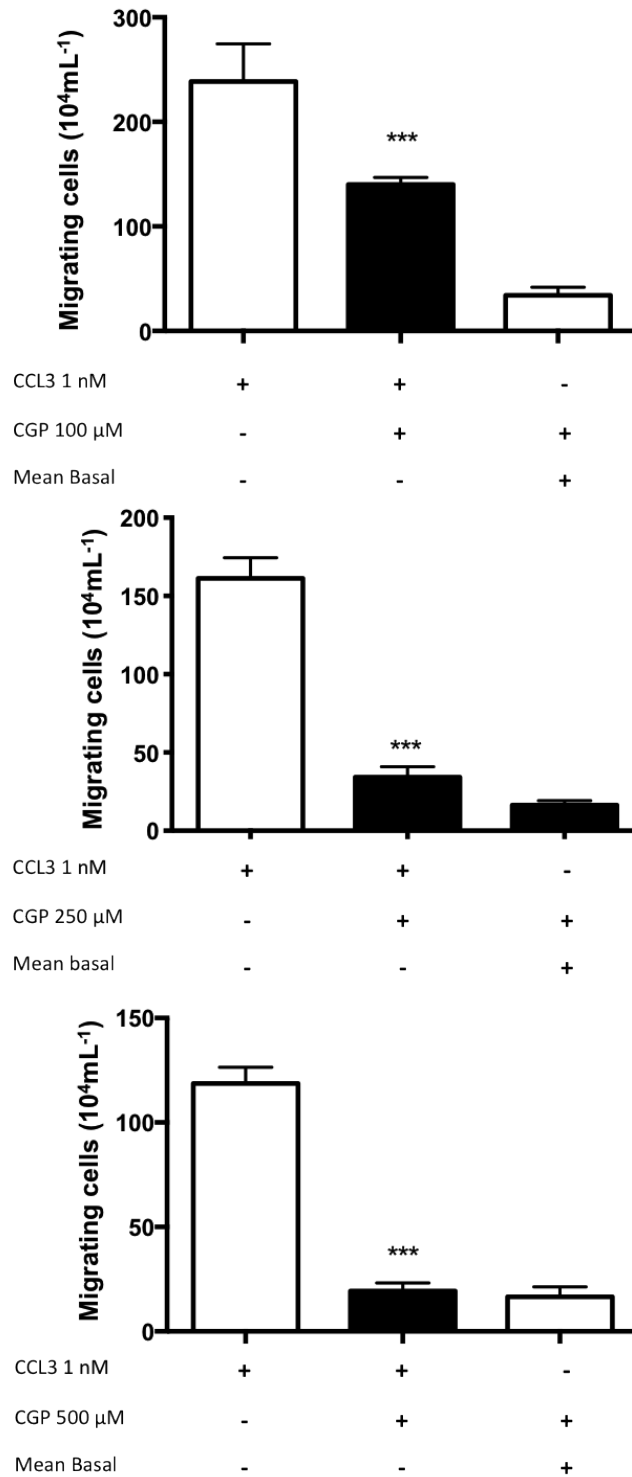


Figure 6.30: Grb2 SH2 domain inhibitor CGP78850 significantly reduces THP-1 cell migration towards CCL3. (a) 100 μM CGP78850 (CGP) significantly reduces THP-1 migration towards 1 nM CCL3. Inhibition is concentration dependent (b & c) with 500μM CGP reducing migration to basal levels. All results represent the mean ± SEM of at least 3 independent experiments (One way ANOVA, Bonferroni multiple comparison. * indicates p≤0.001 vs. vehicle control).**

Pre-treatment of THP-1 cells for 0.5hr with 100, 250 and 500 μ M CGP78850 for 0.5hr before chemotaxis assay towards 1 nM CCL3 resulted in a significant, concentration dependent reduction in migration ($p \leq 0.001$, $n \geq 3$) (Figure 6.30). At 250 μ M migration was not significantly different from unstimulated basal migration ($p > 0.05$, $n \geq 3$). These data suggest that Grb2 plays a crucial role in CCR5 mediated chemotaxis however any cytotoxic effects of CGP78850 treatment need to be determined before the observations can be attributed to Grb2 inhibition.

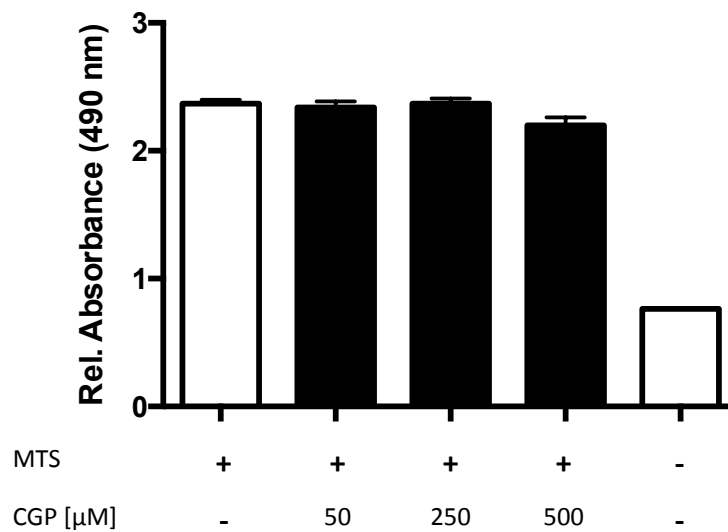


Figure 6.31: CGP78850 does not demonstrate cytotoxicity in THP-1 cells up to 500 μ M. Treatment with increasing concentrations of CGP78850 (CGP) for 0.5hr prior to incubation with MTS for 5hr shows no significant decrease in viability. All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. All treatments not significant = $p > 0.05$).

Pre-treatment of THP-cells with 100, 250 and 500 μ M CGP78850 for 0.5hr prior to incubation with MTS for 5hr demonstrated that the treatment concentrations did not affect cell viability significantly ($p > 0.05$, $n = 3$) (Figure 6.31). These data indicated that the reduction in THP-1 cell migration is not due to cell death, however, there may be other explanations for the observations.

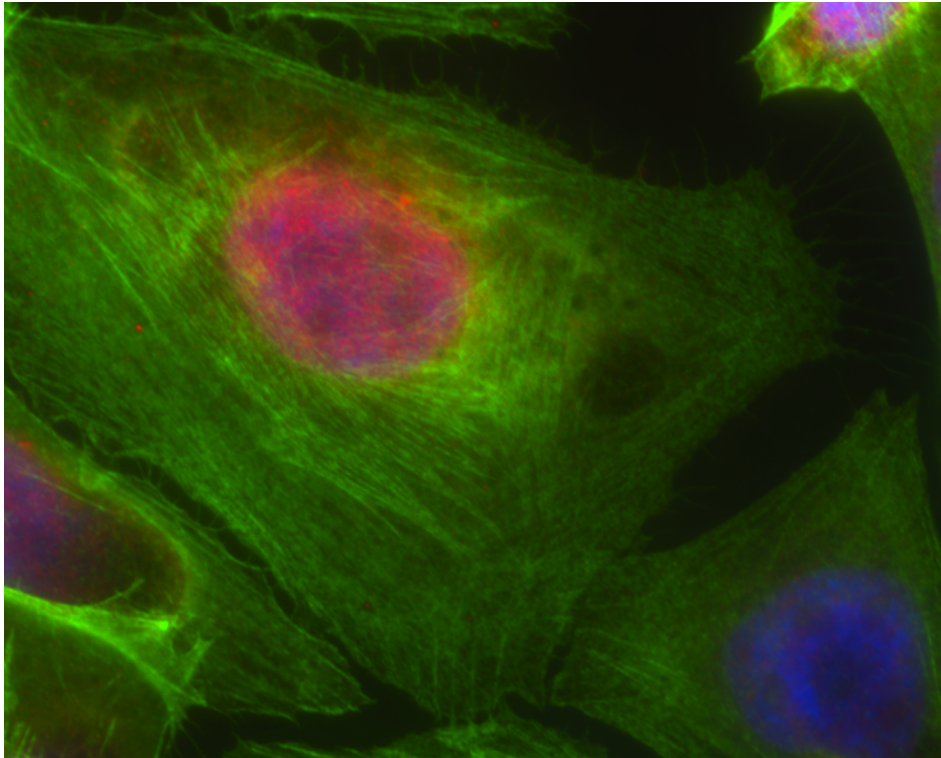


Figure 6.32: CGP78850 does not prevent actin polymerisation in CHO.CCR5 cells. CHO.CCR5 treated with 250 μ M CGP78850 shown no effect on actin stress fiber formation in cells treated with 100 nM for 2hr. Actin stained with Alexa-488 phalloidin (green), anti-dynamin Ab counterstained with Alexa-594 tagged Ab (red) DAPI (blue). Image representative of population, acquired using Zeiss axiovision 2 system.

As observed with the cucurbitacin I, disruption of the actin cytoskeleton can result in a non-specific reduction in migration. So it is important to ascertain if CGP78850 can affect actin stress fiber formation (Figure 6.32). CHO.CCR5 cells were treated with 250 μ M CGP78850 for 0.5hr and then stimulated with 100 nM CCL3 for and stained with Alexa 488 tagged and anti-dynamin 2 Ab and corresponding secondary antibody. Wide field fluorescence microscopy revealed that CGP78850 treatment does not affect the ability of CHO.CCR5 cells to form actin stress fibers nor does it noticeably affect dynamin localisation.

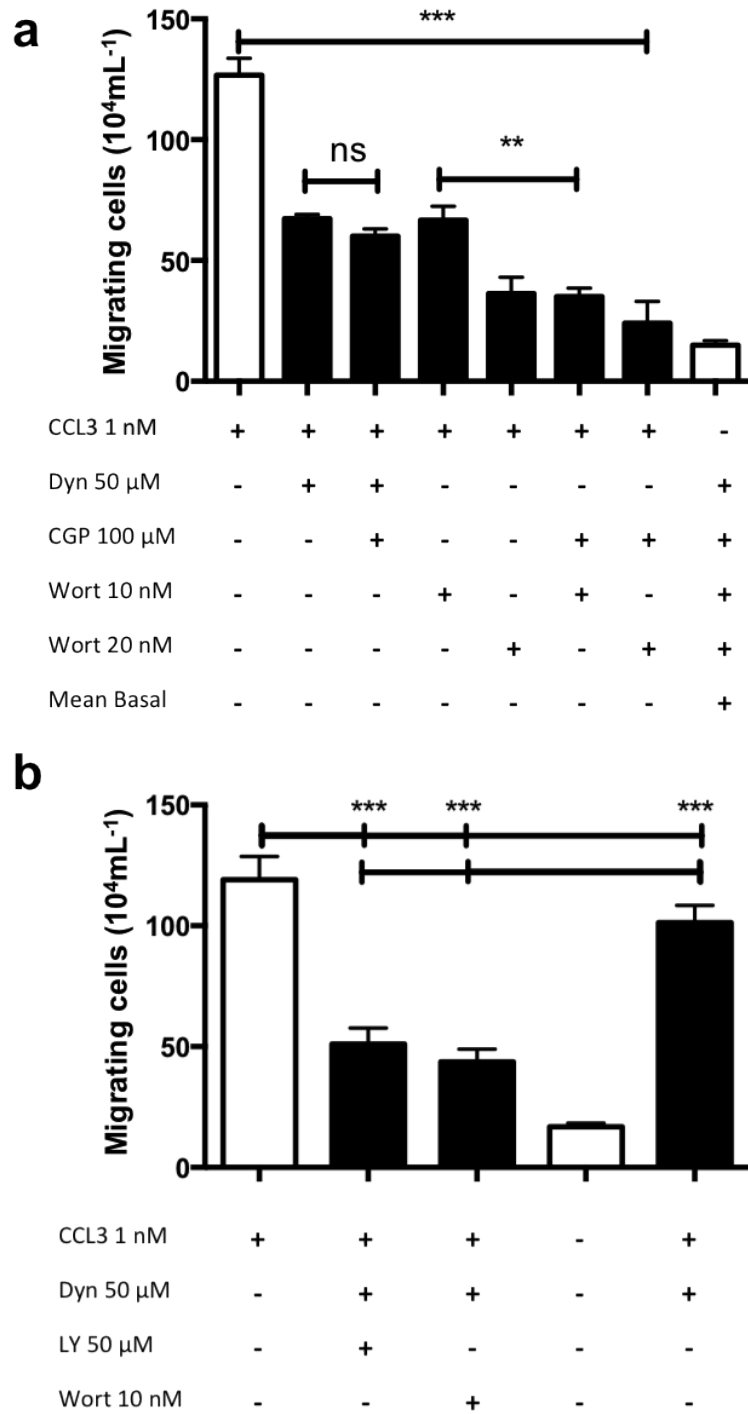


Figure 6.33: Dynasore and CGP87750 increase potency of PI3K inhibitors but not each other.

(a) CGP78850 significantly increases potency of wortmannin (Wort) as an inhibitor of THP-1 migration but not that of dynasore ($p \leq 0.01$ $n=3$). (b) Dynasore increases potency of PI3K inhibitors LY294002 (LY) and Wort ($p \leq 0.001$ $n=3$). All results represent the mean \pm SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison, *** = $p \leq 0.001$).

To investigate the role of Grb2 and dynamin inhibitors further, THP-1 cells were simultaneously treated with different combinations of dynamin, Grb2 and PI3K inhibitors to determine if the potency of these inhibitors can be enhanced in the presence of one another. THP-1 treated with 50 μ M dynasore resulted in a \approx 50% reduction in migration ($p < 0.001$, $n \geq 3$ vs. vehicle), however, simultaneous treatment with 50 μ M dynasore and 100 μ M CGP78850 showed no significant reduction in migration ($p > 0.05$, $n \geq 3$ vs. dynasore 50 μ M treatment) (Figure 6.33a). These data indicate that the combined inhibitory effects of dynasore and CGP78850 are not cumulative, which suggests the molecules function downstream of one another. Cumulative inhibition was seen with the simultaneous treatment of THP-1 cells with 100 μ M CGP78850 and 10 nM wortmannin, which significantly reduces THP-1 migration compared to 10 nM wortmannin alone ($p \leq 0.01$, $n \geq 3$). The reduction in migration was not significantly different to 20 nM wortmannin pre-treatment ($p > 0.05$, $n \geq 3$) which demonstrates that combined treatment effectively doubled the potency of wortmannin. 100 μ M CGP78850 pre-treatment in conjunction with 20 nM wortmannin shows a non-significant trend towards decrease in migration vs. 20 nM wortmannin alone ($p > 0.05$, $n \geq 3$), however, this combined treatment is also not significantly different from the unstimulated basal migration ($p > 0.05$, $n \geq 3$). Dynasore was also used in conjunction with PI3K inhibitors (Figure 6.33b). Pre-treatment of THP-1 with 50 μ M dynasore was used as a baseline and significantly reduces migration ($p \leq 0.001$, $n = 3$ vs. vehicle). Combined treatment with 50 μ M and the PI3K inhibitors LY294004 (50 μ M) and wortmannin (10 nM) significantly reduced migration below this baseline ($p \leq 0.001$, $n = 3$ vs. 50 μ M dynasore pre-treatment). Interpretation of these observations needs to be carefully considered.

Combined treatment of CGP78850 with MiTMAB and OcTMAB was used to determine if the observations with combined CGP78850/dynasore pre-treatment were maintained.

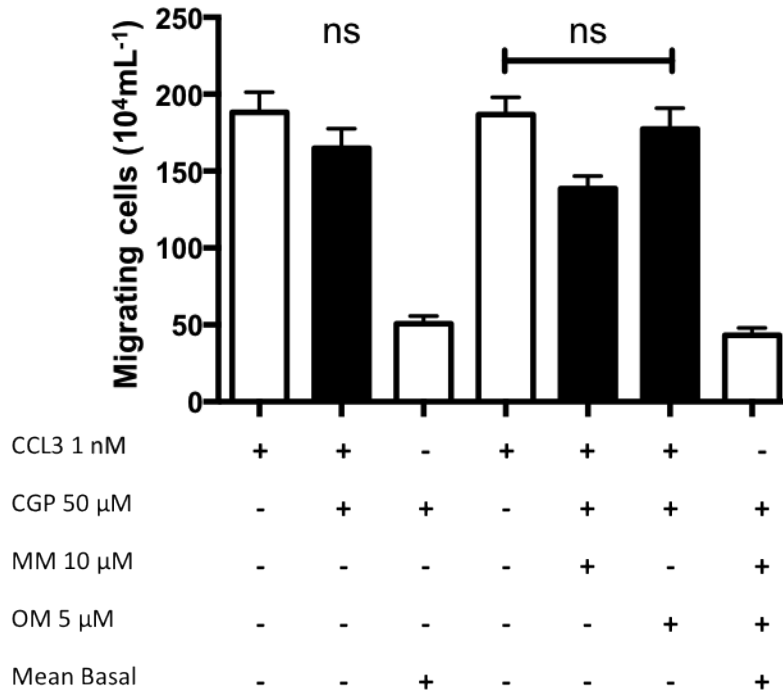


Figure 6.34: CGP78850 does not increase potency of MiTMAB or OctMAB as inhibitors of CCR5 mediated THP-1 cell chemotaxis. Treatment of THP-1 with 50 μ M CGP78850 (CGP) in combination with 10 μ M MitMAB (MM) and 5 μ M OctMAB shows no significant reduction in THP-1 chemotaxis towards 1 nM vs. CGP alone or the vehicle control (n=3 ns= not significant). All results represent the mean \pm SEM of at least 3 independent experiments. (One-way ANOVA, Bonferroni multiple comparison).

Pre-treatment of THP-1 cells with 50 μ M CGP78850 is unable to significantly reduce migration towards 1 nM CCL3 ($p > 0.05$, $n = 3$ vs. vehicle control) and combined treatment with 50 μ M CGP78850 and MiTMAB (10 μ M) and OctMAB (5 μ M) also showed no significant reduction in migration vs. the vehicle control ($p > 0.05$, $n = 3$) (Figure 6.34). These data support the idea that dynasore specific interactions with the PRD interfere with the conformation of GRB2, which would not be expected to occur with the PH domain binding long chain ammonium ion inhibitors.

6.3.6. Signalling to chemotaxis through CCR5 does not involve transactivation of receptor tyrosine kinases

Grb2 inhibition appeared to be able to reduce CCR5 signalling to migratory machinery however there are well established signal transduction pathways involving Grb2 which first need to be investigated before the observations can be attributed to disruption of CCR5 signalling. Cytokine receptor signalling is known to involve signal transduction through Ras, Sos and Grb2 to PI3K. This pathway may be relevant, because there is evidence to suggest that chemokine receptors can transactivate cytokine receptors [85, 96]. Although other observations suggest transactivation is not a likely explanation: for example the insensitivity to PI3K inhibition, the fact that PKC ζ inhibition shows no significant effect and the sensitivity to non-receptor tyrosine kinase inhibition with bosutinib. Inhibition of Ras will provide definitive evidence for the involvement of transactivation. If treatment with a Ras inhibitor is unable to significantly reduce migration, then this will provide strong evidence for at least a partial involvement of transactivation.

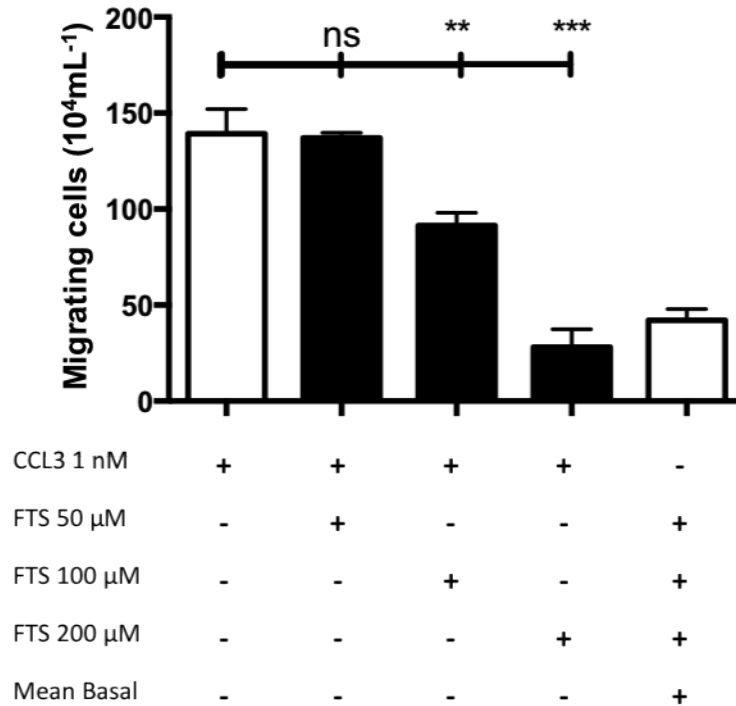


Figure 6.35: Ras inhibitor farnesylthiosalysilic acid significantly reduces THP-1 cell migration above 100 μM. Treatment of THP-1 for 0.5hr with increasing concentrations of farnesylthiosalysilic acid (FTS) prior to migration assay reveal significant inhibition at and above 100μM ($p \leq 0.001$ $n \geq 3$). All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. ** indicates $p \leq 0.01$ vs. control & * = ≤ 0.001 , ns = not significant).**

Farnesylthiosalysilic acid is small molecule inhibitor of Ras which prevents Ras binding to the cell membrane and hence rendering it non-functional (Figure 6.35). The inhibitor has a low μM IC_{50} in isolated membrane systems, but due to poor membrane permeability must be used at concentrations between 25 and 100 μM in whole cells [272]. THP-1 cell pre-treatment for 0.5hr with 50, 100 and 200 μM farnesylthiosalysilic acid demonstrate that concentrations of 100 μM and above could significantly reduce migration towards 1 nM CCL3 ($p \leq 0.01$, $n=6$ vs. vehicle control).

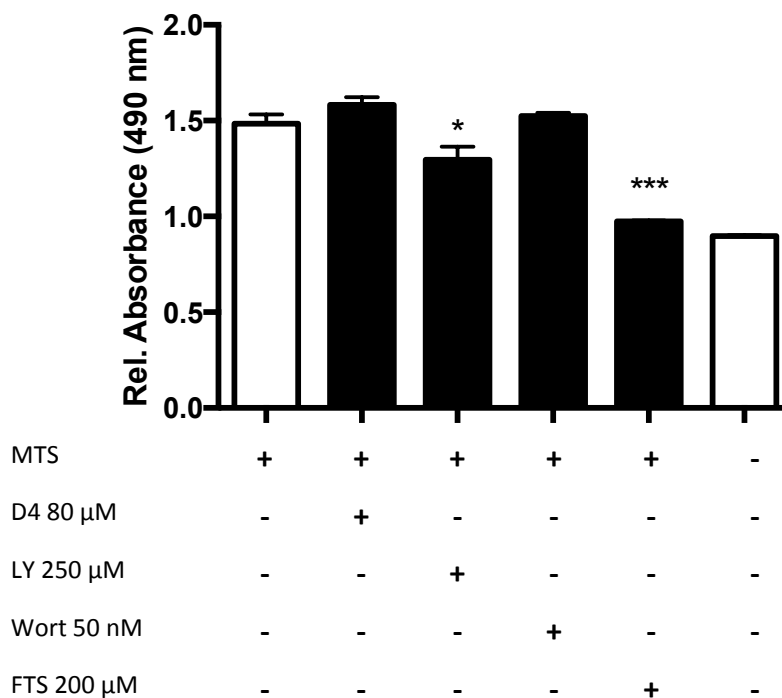


Figure 6.36: Farnesylthiosalysilic acid shows high cytotoxicity at 200 μM. Cytotoxicity assay of various inhibitors used in this chapter reveals farnesylthiosalysilic acid (FTS) significantly reduces viability in THP-1 ($p \leq 0.001$ $n=3$). LY294002 (LY) shows significant cytotoxicity at 250 μM ($p \leq 0.05$ $n=3$). All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.05$ vs. control & * = $p \leq 0.001$).**

The effects of farnesylthiosalysilic acid are likely due to cytotoxic properties as 200 μM treatment is able to completely prevent THP-1 cell viability ($p \leq 0.001$, $n=3$) (figure 6.36). These data suggest that the reduction in migration observed with 100 μM farnesylthiosalysilic acid treatment could be due to cell cytotoxicity rather than inhibition of Ras. This idea is supported by the fact that 50 μM pre-treatment shows no effect on migration, despite being at sufficiently high concentration according to the literature [272]. These data combined with the other relevant observations provide strong evidence that Grb2 inhibition is affecting CCR5 signalling directly and not as a result of transactivated cytokine receptor signalling.

As described in chapter 4, it is important to translate the observation made in THP-1 to primary human tissue to determine if the conclusions are limited to cell type or if they show receptor specific function across cell types. Moving the experimental procedures into activated PBLs also allows comparisons to be drawn against α -chemokine receptors to determine if the proposed signalling mechanism is conserved in other chemokine receptor families.

6.3.7. Inhibition of dynamin in activated peripheral blood lymphocytes

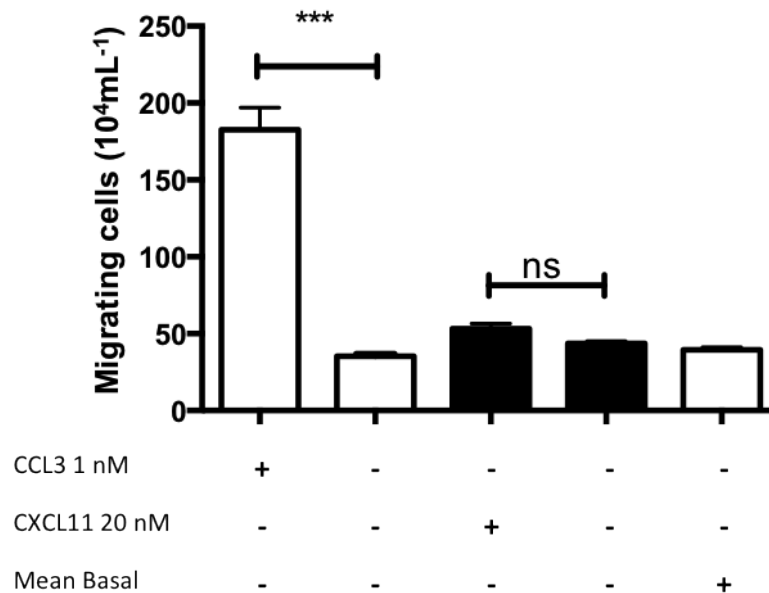


Figure 6.37: THP-1 cells do not migrate towards CXCR3 agonist CXCL11. THP-1 cells show significant migration towards 1 nM CCL3 however migration toward 20 nM CXCL11 is not significantly different from basal unstimulated migration ($p \leq 0.001$ $n \geq 3$) All results represent the mean \pm SEM of at least 3 independent experiments (One-way ANOVA, Bonferroni multiple comparison. *** indicates $p \leq 0.001$ vs. control, ns = not significant).

The α -chemokine receptor ligand CXCL11 and has a sole receptor CXCR3 which is known to be expressed in IL2 activated PBLs. To determine whether THP-1 cells expressed functional CXCR3 receptors untreated cells were stimulated with 20 nM CXCL11. Positive control cells showed normal migration but 20 nM CXCL11 was unable to produce a significant migration in THP-1 cells (Figure 6.37) ($p>0.05$, $n=6$ vs. unstimulated cells). Although the absence of CXCR3 receptor from THP-1 cells cannot be ruled out with these data, they do indicate that there is no functional signalling to chemotaxis via this receptor.

Activated PBLs derived from peripheral blood mononuclear cells are known to up regulate CXC and CC motif chemokine receptor expression and therefore make an ideal primary human model for the observation seen with THP-1 cells. PBLs were stained with anti-CCR5 antibody (Figure 6.38a) and showed expression of the receptor (Figure 6.38a'). CCR5 expressed in these cells seems to be functional as PBLs can migrate towards 1 nM CCL3 ($p\leq 0.001$, $n=3$ unstimulated vs. stimulated). It should be noted that this migration might be mediated through CCR1 which is also present on some activated PBLs [273]. This migration can be completely abrogated by pre-treatment of PBLs with 80 μ M dynasore for 0.5hr prior to chemotaxis assay ($p>0.05$, $n=3$ non-stimulated vs. stimulated) as seen with THP-1 cells. PBLs also migrated towards 20 nM CXCL3 ($p\leq 0.001$, $n=3$ vs. unstimulated controls) indicating functional CXCR3 receptors are expressed. These data indicate that CCR5 function in relation to dynamin inhibition appears to be conserved suggesting that the observations made in THP-1 cells are not cell line specific.

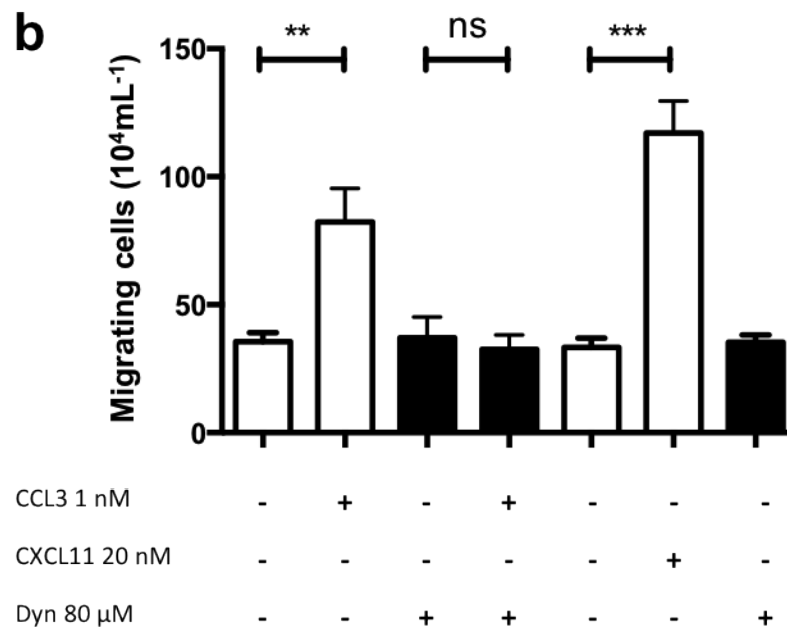
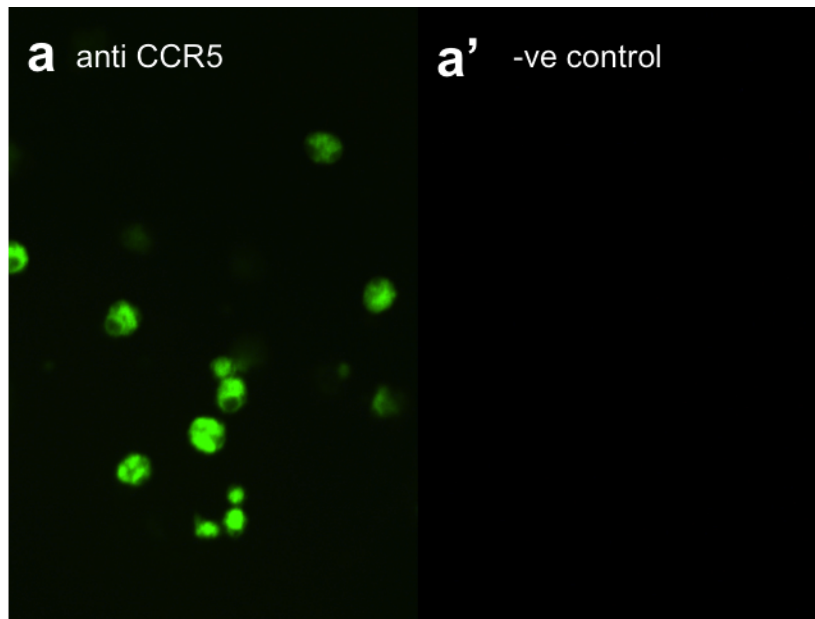


Figure 6.38: Activated peripheral blood lymphocyte migration towards CCL3 is dynasore sensitive. (a) Activated peripheral blood lymphocytes (PLBs) show expression of CCR5 after 10 days incubation with IL-2 and concanavalin-A. Anti-CCR5 stain counterstained with Alexa-488 tagged Ab. (a') secondary control. (B) PBLs show significant migration towards 1 nM CCL3 which is inhibited by 0.5hr pre-incubation with 80 μM dynasore (dyn) ($p \leq 0.01$ $n=3$). PBLs significantly migrate towards 20 nM CXCL11 ($p \leq 0.001$ $n=3$). All results represent the mean \pm SEM of at least 3 donor's PBLs where $n=1$ is the mean of 3 independent experiments for 1 donor (One-Way ANOVA, Bonferroni multiple comparison. ** indicates $p \leq 0.01$ & *** ≤ 0.001 vs. unstimulated control, ns = not significant).

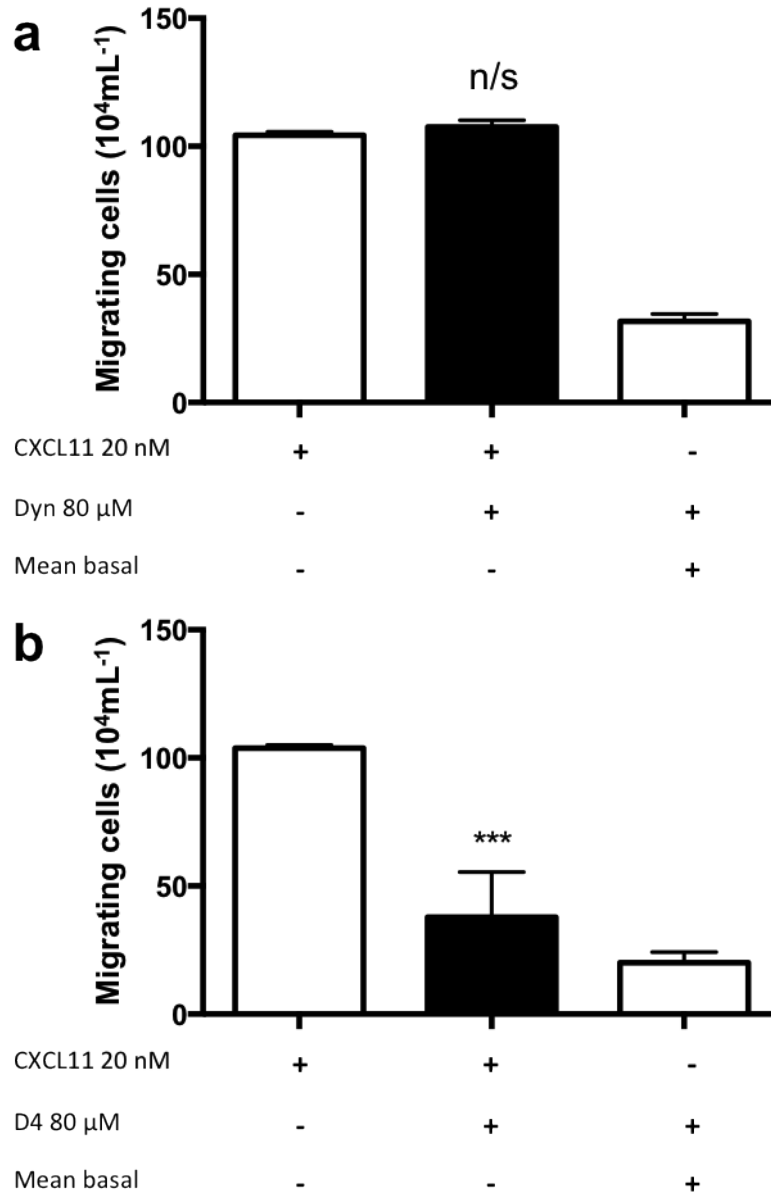


Figure 6.39: Activated peripheral blood lymphocyte migration towards CXCL11 is not sensitive to dynasore but is inhibited by dyngo-4a. (a) Treatment of activated PBLs with 80 μ M dynasore (Dyn) has no significant effect on migration towards 20 nM CXCL11 (n=3). (b) Pre-treatment with 80 μ M dyngo-4a (D4) significantly reduces PBL migration ($p \leq 0.001$ n=3). All results represent the mean \pm SEM of at least 3 donor's PBLs where n=1 is the mean of 3 independent experiments for 1 donor (One-Way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.01$ vs. unstimulated control, ns = not significant).**

Interestingly, pre-treatment of PBLs with 80 μ M dynasore did not block PBL migration towards 20 nM CXCL11 (Figure 6.39a) ($p>0.05$, $n=3$ vs. untreated control), which is in stark contrast to the results observed with CC-chemokine receptor stimulation in PBLs and THP-1 cells. Pre-treatment with 80 μ M dyngo-4a results in a significant reduction in PBL migration towards 20 nM CXCL11 stimulation ($p\leq 0.001$, $n=3$ vs. stimulated control).

Experimental results presented in chapter 4 indicate that PBL chemotaxis is sensitive to gallein treatment, whereas THP-1 cell chemotaxis via CCR5 is not. This suggests that CXCR3 signal transduction to migration occurs via the $G\beta\gamma$ mediated route, unlike the CCR5 signalling network. It is therefore important to ascertain whether there are other differences in signalling between CCR5 and CXCR3, which can be identified pharmacologically and may allow a clearer picture of the CCR5 specific signalling events seen in THP-1 cells. THP-1 cells have a low sensitivity to PI3K inhibitor LY294002 and are unaffected by PI3K γ inhibitor AS605240. There is evidence that small highly mobile cells such as monocytes do not require PI3K for polarisation in migration [115] in the same way as larger adherent cells do, but there is also evidence to suggest that T-cells do require PI3K [248]. To determine if the observation made in THP-1 cells with regard to PI3K inhibitors was receptor or cell specific, PBLs were pre-treated with LY294002 (125 μ M) and AS605240 (1 μ M).

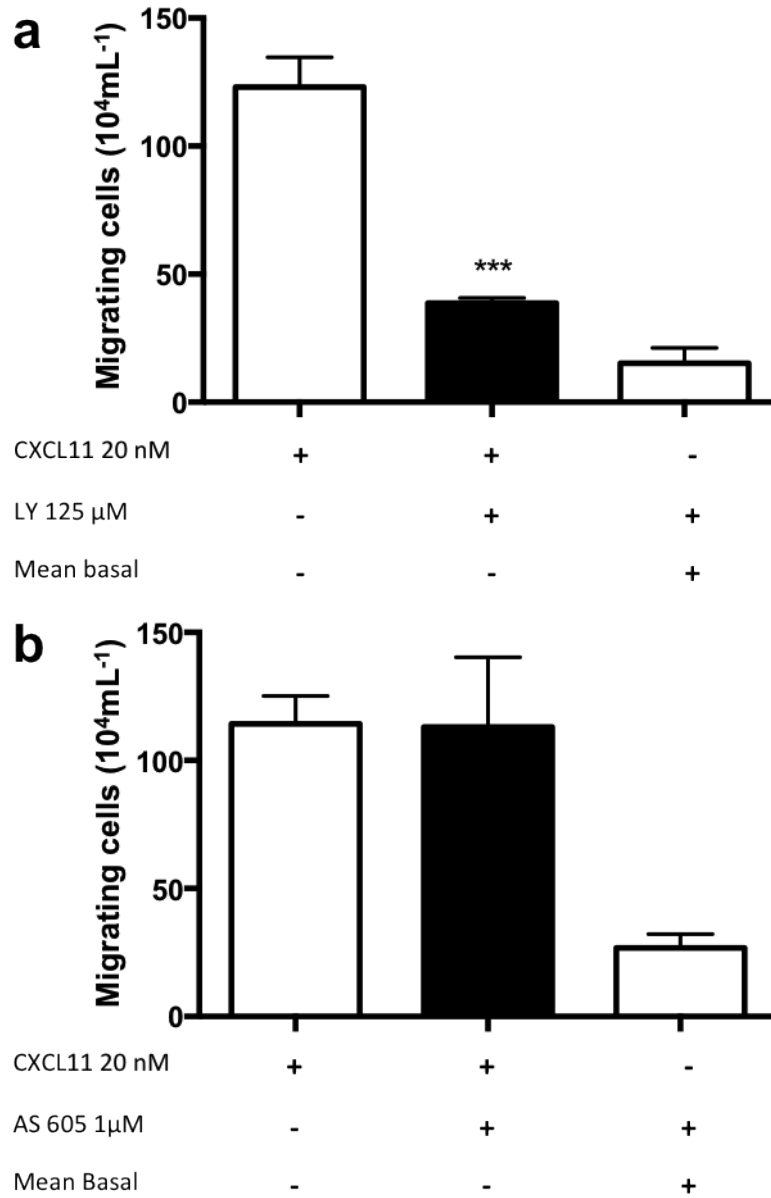


Figure 6.40: PBL migration is sensitive to LY294002 but not AS605240 inhibition. (a) Pre-treatment of activated PBLs with 125 μ M LY294002 (LY) significantly reduces migration towards 20nM CXCL11 ($p \leq 0.001$ $n=3$). (b) 1 μ M AS605240 (AS605) has no significant effect ($n=3$). All results represent the mean \pm SEM of at least 3 donor's PBLs where $n=1$ is the mean of 3 independent experiments for 1 donor (One-Way ANOVA, Bonferroni multiple comparison. * indicates $p \leq 0.01$ vs. unstimulated control).**

PBLs pre-treated with 125 μ M LY294002 for 0.5hr prior to chemotaxis assay show significantly reduced migration towards CXCL11 (Figure 6.40a) ($p \leq 0.001$, $n=3$ vs. stimulated control). Considering the normal inhibitory concentrations for LY294002 are 12 fold lower than those used, these data indicate that PBLs also show low sensitivity to LY294002, which may also represent off-target inhibition of PIM1 or other non-PI3K proteins. This data is supported by the fact that 1 μ M AS605240 pre-treatment did not significantly alter PBL migration (Figure 6.40b) ($p > 0.05$, $n=3$ vs. stimulated control).

The Grb2 inhibitor CGP78850 was used to pre-treat PBLs at a concentration of 100 μ M which has been shown to significantly decrease migration in THP-1 cells (Figure 6.41a). Treated PBLs showed no significant decrease in migration vs. the untreated control cells ($p > 0.05$, $n=3$) indicating that Grb2 is not important in CXCR3 mediated chemotaxis. It is therefore unlikely that Ras is involved directly or through transactivation downstream of CXCR3. Pre-treatment with 100 μ M farnesylthiosalicylic acid did not significantly reduce PBL migration in response to 20 nM CXCL11 ($p > 0.05$, $n=3$) supporting this idea.

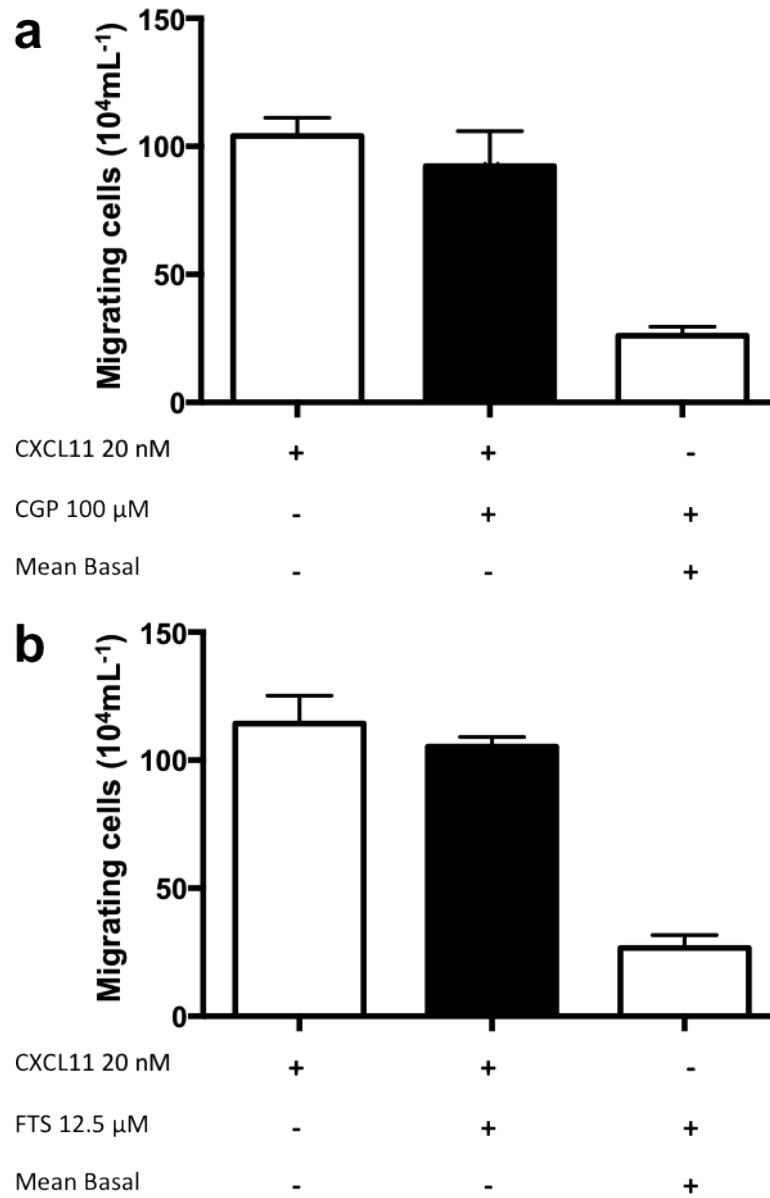


Figure 6.41: CGP87750 and farnesylthiosalysilic acid have no significant effect on activated PBL chemotaxis. (a) Pre-treatment of activated PBLs with 100 μ M CGP78850 (CGP) has no significant effect on PBL migration towards CXCL11 (n=3). (b) 12.5 μ M farnesylthiosalysilic acid also has no significant effect (n=3). All results represent the mean \pm SEM of at least 3 donor's PBLs where n=1 is the mean of 3 independent experiments for 1 donors PBLs. (One-Way ANOVA, Bonferroni multiple comparison).

6.4. Discussion

6.4.1. Internalisation and the possible relationship to arrestin dependent signalling

The primary objective for this chapter was to explore the questions raised in chapter 4 regarding the role of endocytosis machinery, cytoskeletal mediators and polarisation on CCL3 mediated THP-1 cell migration. In particular, the role of arrestins needed to be expanded by examining the relationship between internalisation and migration. It was also important to further resolve the possible mechanisms by which non-receptor tyrosine kinase signalling during migration links to chemokine receptor activation.

CCR5 has been shown to internalise by both clathrin and caveolin mediated endocytosis pathways [213]. These processes can be inhibited by hyperosmolar sucrose [178] and filipin [74] treatment respectively and have been validated to prevent internalisation in THP-1 [178, 213]. Treatment of THP-1 cells with sucrose and a combination of sucrose and filipin demonstrated that the physical process of internalisation is not required for migration. Interestingly, combined sucrose/filipin treatment demonstrated a non-significant trend of more cells migrating towards a stimulus. This may be explained by an increase in arrestin dependent signalling, which is allowed by the stalled CCP and caveolae. Although arrestins do not associate with caveolae, dynamin does and the observed increase in migration may represent enhanced dynamin function at the stalled caveolae. These results were confirmed with the small molecule inhibitor of clathrin: pit stop 2 [260]. Pit stop 2 inhibits clathrin coated pit formation by low affinity binding to clathrin, which has been validated in numerous cell lines. Immunofluorescence validated the effect of pit stop 2 in CCR5 internalisation, but more interestingly demonstrated, that THP-1 cells treated with pit stop 2, significantly increased migration by 30%. The observed increase in chemotaxis supports the idea that internalisation is negatively associated with migration. Arrestin dependent internalisation and signalling are also known to occur independently of each other [35, 66] and depend on differential post transcriptional phosphorylation and ubiquitination [63]. In summary, this observed increase in migration may

represent a shift towards arrestin dependent signalling (ADS). If arrestin 'fate' to internalisation or ADS scaffolds is in an equilibrium state determined by interactions cytoplasmic signalling complement then preventing internalisation would be expected drive the equilibrium towards ADS (Figure 6.42). The observations made with PKC and GRK2 inhibitors in chapter 4 support these observations and suggest not only that CCR5 mediated migration in THP-1 is arrestin dependent but also that there are numerous routes to modulate this signalling. The exact mechanism by which this shifting in arrestin signalling can occur will need to be the subject for future investigation. Whether arrestins coordinated with internalisation machinery can have their signalling fate changed by disassociation of this machinery and re-association of ADS machinery remains to be determined but would offer an explanation for the observed effect of internalisation and arrestin recruitment inhibitors on migration. Being able to shift cellular responses based on arrestin function clearly has therapeutic potential for anti-migratory therapies and demonstrates that ligand biased signalling can be artificially induced.

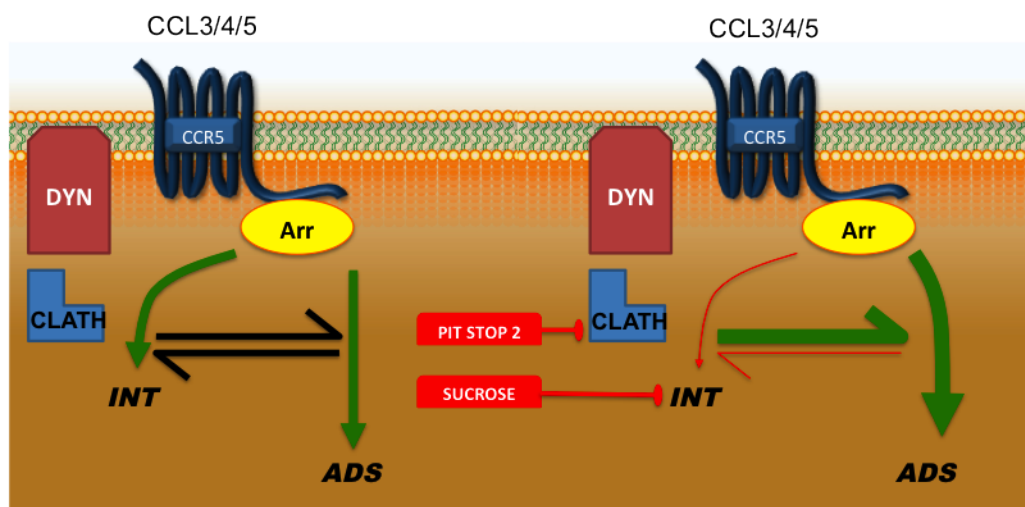


Figure 6.42: A possible mechanism by which inhibition of endocytosis leads to increased migration in THP-1 cells. Under normal conditions arrestin signalling to internalisation (INT) and arrestin dependent signalling (ADS) proteins is in some kind of equilibrium based on either cytoplasmic protein complement or arrestin recruitment protein function. Stalling internalisation drives the equilibrium towards arrestin dependent signalling by disassociation of internalisation machinery and association of signalling scaffolds.

6.4.2. PI3K mediated cell polarisation is not required for CCR5 mediated chemotaxis in THP-1 cells

Chemotaxis involves movement of a cell towards an extracellular gradient of stimulant. Intracellular gradients of signalling proteins are also formed in response to receptor activation in a process called polarisation. Polarisation allows the intracellular machinery of a migrating cell to function in a concerted effort producing motility towards the stimulus and is considered essential to chemotaxis [115] in large adherent cells. Polarisation is generally believed to occur through phosphorylation of phosphoinositides by PI3K at the leading edge of the cells [76]. Phosphatases such as PTEN and SHIP localise away from the leading edge of the cells and down regulate the products of PI3Ks, which results in internal membrane bound gradients of PIP2 and PIP3 that are focused around the leading edge of the cells. This internal polarisation allows the targeting of the relevant cytoskeletal mediators to the leading edge where they can exert motile efforts in a direction of stimulant gradient, but also allow the disassembly of focal adhesions at the 'tail end' of migrating cell. Rho family kinases are crucial for focal adhesion formation and formation and disassembly of other membrane protrusions [108] and chapter 4 did show, that this part of THP-1 chemotaxis functions in a predictable way. The evidence from chapter 4 suggests that non-classical signalling is occurring at the level of receptor transduction to the upper echelons of chemotaxis signalling in THP-1. It is therefore important to determine which characteristics of G $\beta\gamma$ -type migration CCR5 mediated responses shared and hence, the effect of PI3K inhibition on THP-1 chemotaxis was characterised. This is particularly interesting because Volpe *et al.* propose that polarisation in THP-1 does not occur via PIP2 and PIP3 production by PI3K [115]. Volpe *et al.* however did not approach the observed lack of normal polarisation pharmacologically, that is to test their claims with PI3K inhibitors, nor did it determine the receptor specificity of their observations. As stated in the introduction there is a difference between identifying a protein target and that target being pharmacologically accessible. The use of small molecule inhibitors of PI3K in THP-1 chemotaxis assay may support the assertions of Volpe *et al.* evidence from a 'drug' but also determine if their observations are true for CCR5 mediated chemotaxis.

Both LY294002 and wortmannin pre-treatment affects THP-1 migration towards CCL3, but at concentrations much higher than seen in the literature [274] and well beyond their determined IC_{50} values for PI3K inhibition [116, 117]. Both inhibitors are known to inhibit other proteins [261-263] at higher concentration below those seen to be efficacious against THP-1 migration, so the possibility that the reduction in migration was a result of inhibition of these secondary targets is real. To resolve whether or not LY294002 and wortmannin are acting through PI3K or their secondary targets, the rationally designed PI3K γ inhibitor AS605240 was also used to pre-treat THP-1. GPCR signalling via G $\beta\gamma$ is thought to occur predominantly through PI3K γ [110, 118, 216] and to a lesser extent through PI3K δ , so the inhibitor AS605240, which was designed to have a higher affinity for PI3K γ isotype [118] and has no known secondary targets, was used to determine if PI3K is required for migration. Treatment of THP-1 cells with AS605240 at a concentration of 1 μ M, sufficient to inhibit all PI3K isotypes, has no significant effect on migration. Partial effects can only be seen at concentrations at about 10 μ M, which is of sufficiently high concentration that non-specific or cytotoxic effects could play a role. AS605240 has been shown to reduce PKB phosphorylation in THP-1 at 100 nM [216]. These data as a whole support, but also pharmacologically validate, the findings of Volpe *et al.* [115] and show that CCR5 mediated migration in THP-1 does not differ from β -Adrenergic receptor signalling in this regard. These data also bolster the evidence that CCR5 signalling to chemotaxis in THP-1 is G $\beta\gamma$ independent, which represents an original observation. The activation of PI3K γ downstream of G $\beta\gamma$ is the route by which signalling to G $\beta\gamma$ dependent migration is thought to occur [57].

6.4.3. A pharmacological investigation of dynamin inhibitors in CCR5 mediated THP-1 chemotaxis

The small molecule inhibitor of dynamin dynasore was initially used to further explore the role of internalisation on migration. Interestingly, this inhibitor proved to have high potency in blocking THP-1 migration. This was surprising initially due to the observation made with PKC, GRK2 and internalisation inhibitors however dynamin is also known to play a role in migration

as described in the introduction [77, 135]. Dynamin is known to interact with both G $\beta\gamma$ [199] and arrestin [65], so it seems plausible that dynamin forms part of the arrestin dependent signalling complex to migration in THP-1. Gallein treatment had not affected THP-1 migration, suggesting, that dynamin-G $\beta\gamma$ interaction is not crucial for migration and other interactions should be given priority for investigation.

In order to gain more information about cellular activation, a calcium release assay was also used to investigate the effect of dynasore, despite the fact that calcium release and migration are independent in this cell line [75]. Dynasore completely blocks calcium release stimulated by CCL3, which was surprising but not without precedent [201]. The reduction in calcium release and migration is not a result of cytotoxicity, which made dynasore the first inhibitor to link a reduction in calcium with migration in THP-1 cells. The presence of dynamin was validated in the model cell lines and internalisation of CCR5 receptors in HeLa.RC49 is blocked by treatment with the inhibitor. These data together with the already published validation of dynasore in THP-1 cells in the literature by other groups [134, 178] suggest that the observations are indeed a result of dynamin inhibition.

Dynamin has been shown to regulate actin fibre polymerisation and localisation but treatment of CHO.CCR5 cells with dynasore did not have the obviously disruptive effects on actin polymerisation seen with cytochalasin D or cucurbitacin I as seen in chapter 5. This suggests that any effects the dynamin inhibition was having on actin dynamics occur at a more subtle level and this supports others findings, that although actin polymerisation is decreased by dynamin inhibition and K44A, some actin stress fibres can still form in the absence of dynamin GTPase activity [100, 164].

Immunofluorescence shows that dynamin localises at fibrous structures in the cytoplasm which did not stain with phalloidin. These structures were thought to be microtubules, which would fit with what is known about dynamin function [130, 275]. There is some evidence in the literature that the microtubule-actin fibre interactions play a role in migration [76, 102, 267] and to

determine if microtubule polymerisation is required for migration, nocodazole was used to treat THP-1 cells. Nocodazole treatment does not block THP-1 cell chemotaxis, which suggests that dynamin interactions at this locus are not crucial for migration. This observation fits with the literature where dynamin/microtubule interactions are more associated with cell cycle progression [150, 266, 276].

THP-1 cells have been shown to be adversely affected by the electroporation process which limited the scope for mutant arrestin transfection in this model of migration. However K44A transfection can still be useful to evaluate the effect of dominant negative mutant over expression on calcium release. THP-1 cells transfected with K44A mutant dynamin show non-significant alteration in calcium release compared to control cells which indicates that the effects of dynasore on calcium release are either due to functions of dynamin which are not dependent on GTPase turnover or are a result of non-specific interaction specific to the molecule.

The recent spate of dynamin inhibitors which have been described earlier offer a unique opportunity to investigate the function of specific functional domains of the protein whilst allowing comparative analysis of other factors such as GTPase and internalisation efficacy. The dynamin inhibitors MiTMAB, OcTMAB, dynole 34-2 and iminodyn 22 have a range of dynamin GTPase inhibitory potencies that are higher than that of dynasore and were used to treat THP-1 cells at concentrations above these IC_{50} values. None of the tested compound showed any significant ability to block THP-1 migration at the concentrations used. This was surprising considering the effect of dynasore and the literature which has shown MiTMAB and OcTMAB to reduce migration in some adherent cells. Critically though this experiment shows that the potency these inhibitors have in blocking the dynamin GTPase function does not correlate with the observed effect on migration. Dynasore has a much lower dynamin GTPase IC_{50} than iminodyn 22 and dynole 34-4, but these inhibitors do not reduce migration significantly. The literature has mixed reports on whether dynamin GTPase turnover is required for migration,

with GTPase function thought to be necessary for dynamin mediated actin polymerisation [164] but not to be required for PRD interactions with actin mediators such as cortactin [100].

The effect of dynasore, therefore, may have been a result of steric hindrance of other protein interactions around the GTPase allosteric site that it binds. This is supported by the fact that the uncompetitive inhibitors dynole 34-1 and iminodyn 22, which must bind to the GTPase-GTP complex, limiting potential interaction to this locus distal from the PRD (Figure 6.43), do not inhibit migration. To explore this idea, the dynasore analogue dyngo-4a was used to treat THP-1 cells prior to chemotaxis assay. Dyngo-4a resulted from a structure activity development of dynasore against GTPase activity and differs by only one terminal aryl hydroxyl group but has significantly improved potency against dynamin GTPase turnover [142]. Concentration response curves for dynasore and dyngo-4a revealed that the potencies of dynasore and dyngo-4a against THP-1 migration are 52 and 103 μM , respectively. This observation indicates not only that the GTPase potency has no correlation to the effect the inhibitor has on migration, but also tuning of inhibitors towards higher GTPase efficacy detracts from their potency as inhibitors of migration. These data clearly show that there is scope for structure activity relationship development for dynamin inhibitors which act preferentially on migration rather than GTPase/internalisation. This point is exemplified by the equimolar comparison between dynasore, dyngo-4a and the structurally similar iminodyn 22, which show increasing GTPase inhibition potency, but which demonstrate a clear structure activity relationship for inhibition of chemotaxis. Although other inhibitors also appeared to inhibit migration at 80 μM , this was shown to be due to cytotoxic effects. This observation suggests that inhibition of migration in the literature [149] using MiTMAB and OcTMAB were in cells where dynamin recruitment to phosphoinositides is important or where GTPase function is crucial for migration. The observations made with the dynamin inhibitors also did not correlate to their ability to inhibit receptor mediated internalisation or their ability to stabilise dynamin rings which supports the idea that the effect of dynasore and dyngo-4a is based not on these processes but a structure specific inhibition of other protein interactions, possibly at the PRD where the dynasore analogues closely bind to. The effects of dynasore and dyngo-4a inhibition were also seen in HeLa.RC49 wound healing assay suggesting the effect of dynamin inhibition is relevant between

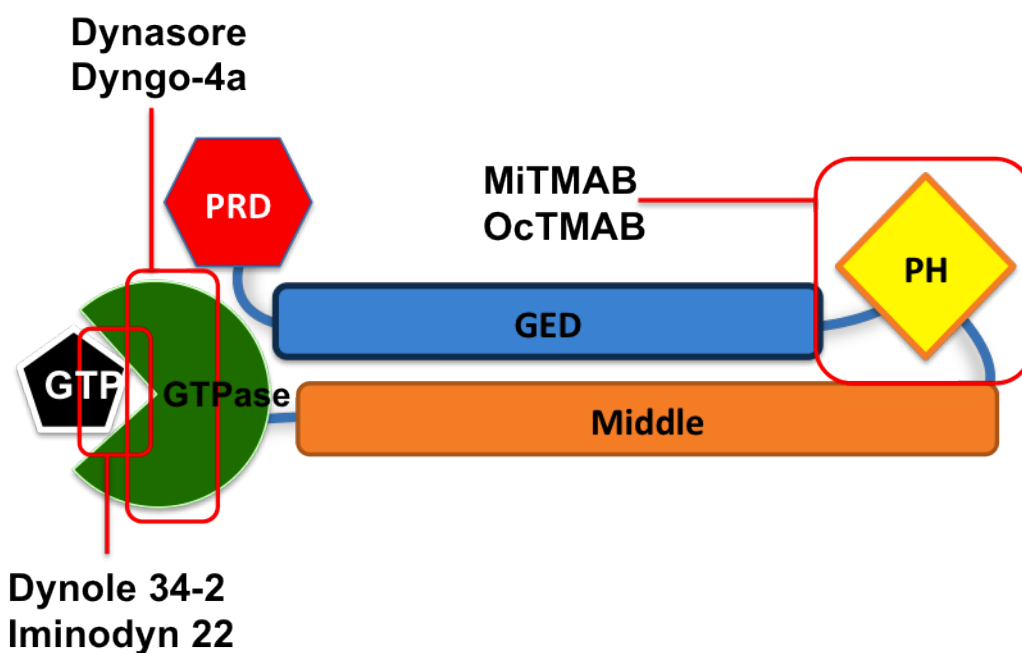
cell types although the difference between transwell and wound healing assay as described in chapter 4 make direct comparison difficult.

Migration towards a scratch was used to examine the effect of dynasore on dynamin localisation during wound healing. As stated previously, dynamin did not affect actin stress fibre formation in CHO.CCR5 and HeLa.RC49 cells and was shown also not to affect the localisation of dynamin at the terminal regions of actin stress fibres in CHO.CCR5 treated for 0.5hr and 24hrs. These cells did however show differences in the way they polarised towards scratches; cells treated for 0.5hr appeared similar to control cells with obvious polarisation towards the wound. Cells treated for 24hr with dynasore, however, were not clearly polarised towards scratches, although they did show numerous membrane extensions around the cells. Cells treated with dynasore for both 0.5 and 24hr also showed a trend towards increased filopodia. These data indicate that dynamin inhibition affects the cells ability to polarise, which is known to be important in fibroblasts and adherent cells but how relevant this is to THP-1 cell migration is unclear as polarisation appears not to occur though the same PI3K dependent route [115]. THP-1 migration is affected by 0.5hr treatment, which suggests high turnover-rate membrane structures such as podosomes [100] are more likely to be involved as opposed to the low turnover focal adhesions seen in large adherent cells. This is supported by the HeLa.RC49 wound-healing assay where sensitivity to LY294002 is high with cells actually detaching after 24hrs. In this system, CCR5 mediated chemotaxis might recruit PI3Ks in order to polarise cells and this also makes possible interaction between dynamin and phosphoinositides such as PIP₂ a possibility.

The function of PH domain recruited dynamin in fibroblast migration may require different dynamin-effector interactions and may even be GTPase turn over dependent which would explain the similar behaviours of dynasore and dyngo-4a in this assay. It should be noted that unusual alterations in cell morphology were noted (not shown) with dyngo-4a, which may also suggest the molecule has some cytotoxicity of non-specific effects at longer incubation times. Taken as a whole these data suggest that dynamin is required for CCR5 mediated THP-1

migration, but that dynamin GTPase turnover is not required. The observations made with bosutinib in chapter 4 suggest that SH2 targeting plays a role on migration, which means that the effects of dynasore and dyngo-4 may have been as a result of disruption of adaptor proteins at the PDR (Figure 6.43). Due to the conformation of dynamin, the PRD is close to the GTPase domain; so allosteric binding of dyngo-4a and dynasore may induce conformational changes in the PRD which can alter its function. It should be noted that dynasore has been shown to have no effect on Grb2 binding, but that does not rule out induction of disruptive interaction with Grb2 [158]. Tyrosine phosphorylation is a recurring theme in dynamin's role in actin regulation [100, 135, 159] and an investigation with SH2 inhibitors was the natural progression.

In the first instance, there were unanswered questions regarding the effect of dynasore on calcium release to be considered. These were investigated by treatment of THP-1 cells with the other dynamin inhibitors. Unlike with chemotaxis, the dynamin inhibitors OcTMAB and dynole 34-2 significantly reduces calcium release, but again there appears to be no correlation with the potency to act as a dynamin GTPase inhibitor or other inhibitory effects. Dynasore, OcTMAB and dynole 34-2 inhibit calcium release in a concentration dependent fashion as would be expected with true CCR5 mediated signalling to calcium release. However, it became immediately apparent that the inhibitors are acting as nonspecific stimulators of calcium release when they reduced thapsigargin stimulated calcium release. This nonspecific release was validated with dynasore, which directly stimulates calcium release in THP-1 cells. These results are interesting but of significantly less importance to the investigation of chemotaxis as they do not contradict the earlier observations of Cardaba *et al.* that calcium release is independent of migration in THP-1 cells [75].



Compound	GTPase IC ₅₀	RME IC ₅₀	Ring Stabiliser ?	THP-1 migration inhibitor?
Dynasore	15 µM	75 µM	✓	✓✓
Dyngo-4a	0.3 µM	16 µM	✓	✓
MiTMAB	3 µM	20 µM	✗	✗
OcTMAB	2 µM	16 µM	✗	✗
Dynole 34-2	1 µM	5 µM	✗	✗
Iminodyn 22	0.4 µM	10 µM	✓	✗

Figure 6.43: Dynamin inhibitor overview. The theorised dynamin inhibitor binding locations and their relationship to inhibition of cellular responses are shown. Values for dynamin inhibition are taken from Table 1.3.

The importance of these results is for other investigators who might be using these compounds to investigate calcium sensitive cellular processes, for example Douthitt *et al.* observed an unexplained increase in neuronal cell calcium after dynasore treatment which these data explain through nonspecific release of calcium [201]. Investigation of endocytosis in calcium

sensitive systems such as neurones will need to be undertaken with caution when using dynasore, OcTMAB or dynole 34-2 treatment as these inhibitors may alter disrupt normal signalling by non-specific calcium release.

6.4.4. Grb2 inhibition and THP-1 chemotaxis

The presence of Grb2 in THP-1 cells was confirmed by western blotting. The protein appears to localise throughout the cytoplasm in HeLa.RC49 cells with no significant alterations in localisation upon CCR5 activation. The small molecule CGP78850 was designed by Novartis to inhibit Grb2 SH2 domains, which made it ideal for investigating the effect of Grb2 SH2 function in THP-1, mediated chemotaxis. CGP78850 is not an ideal drug, due to poor membrane permeability and the ester conjugated prodrug was designed to overcome this [113]. Neither CGP78850 nor the prodrug CGP85793 are commercially available. Novartis were only able to release CGP78850 however and the function of this inhibitor is characterised sufficiently in that cytoplasmic concentrations have been shown to be 20-50 times lower than dose [112]. This meant that although higher concentrations of CGP78850 were used the effective cytoplasmic concentrations were around those expected to inhibit Grb2 without nonspecific effects being a problem. CPG78850 treatment of THP-1 cells resulted in significant reductions in observed migration towards CCL3 that could not be explained by cytotoxicity or disruption of actin stress fibre formation. Combined treatment of THP-1 cells with CGP78850 and dynasore provided evidence that dynamin and Grb2 function in the same linear signalling axis as the combined treatment showed further decrease in migration. This may also support the idea that Grb2 and dynamin interact in a 1:1 stoichiometry. The combined effect of dynasore and CGP78850 with PI3K inhibitors is explained less easily. Combined treatments of dynasore together with wortmannin or LY294002 and CGP78850 with wortmannin or LY294002 reduce chemotaxis further than either treatment alone. This may be a result of disruption of arrestin signalling scaffolds, where PI3K is known to associate or could represent a shift toward PI3K dependent signalling in cells where dynamin and Grb2 are inhibited. The exact mechanism for these reductions requires further investigation before conclusions can be drawn with confidence.

Based on the observations from this and the previous chapter it is possible to propose a hypothetical signal transduction mechanism for CCR5 mediated chemotaxis in THP-1 (Figure 6.44). Upon ligand binding CCR5 undergoes phosphorylation by GRK2 and PKC and a proportion of receptors are bound by arrestins to which the arrestin dependent signalling scaffolds will bind. These scaffolds contain proteins such as ERK, PI3K and non-receptor tyrosine kinases such as Lyn. These non-receptor tyrosine kinases target Grb2 bound dynamin monomers or target SH2 domains of Grb2 which bind to monomeric dynamin and recruit other actin cytoskeleton mediators such as n-WASp and cortactin. Dynamin binding to the Grb2 complex nucleates dynamin polymerisation which acts to uncap actin and promotes actin polymerisation or promotes direct dynamin interaction with actin. This process instigates the formation of membrane protrusions mediated by ROCK, which may be targeted by interaction with dynamin, and promotes migration. In this model PI3K mediated polarisation is not required because arrestin bound non-RTKs act as polarisation indicators.

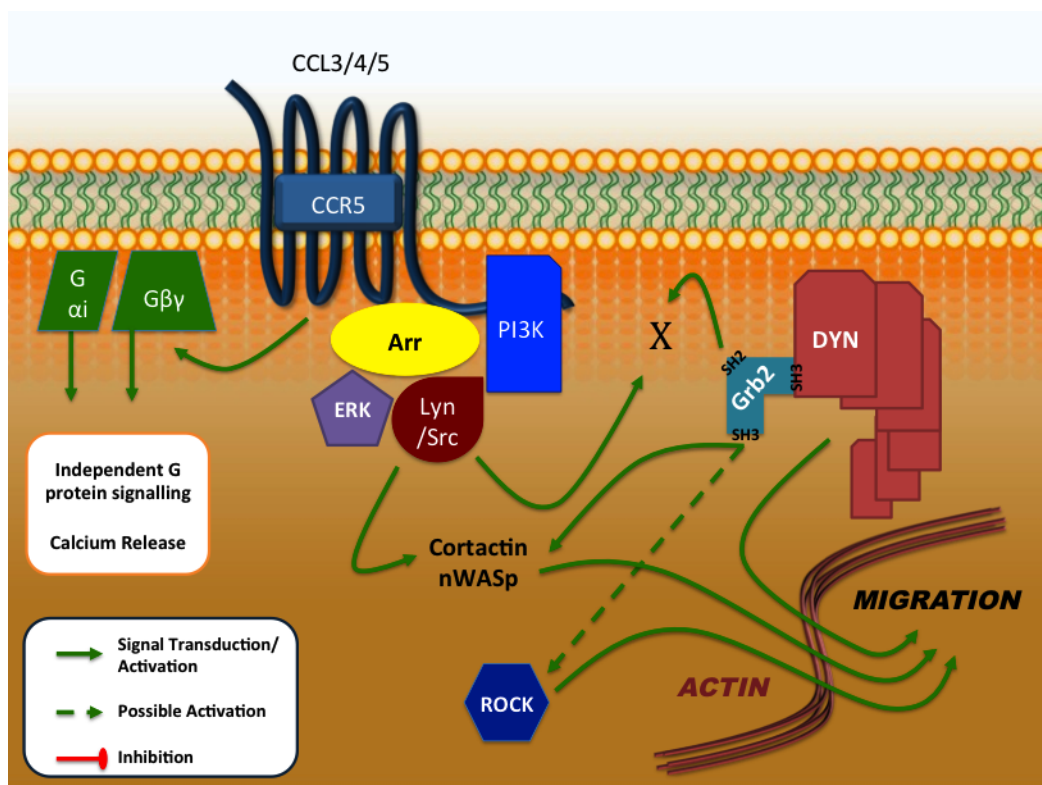


Figure 6.44: CCR5 signalling to chemotaxis in THP-1 cells. This putative signalling diagram is based on results obtained from this thesis and known interaction. The site of non-receptor tyrosine kinase phosphorylation 'X' which may act to target Grb2 bound dynamin is not known.

Whilst Grb2 appears to be involved with CCR5 signalling to chemotaxis, it is important to explore whether the observations can be explained by receptor tyrosine kinase transactivation. CCR5 is known to transactivate RTKs [85] and RTK activation has been linked with migration. Grb2 forms a part of the well characterised signal transduction from RTKs to PKB via Ras [112]. There is good reason to believe that transactivation is not playing a major role in CCR5 mediated chemotaxis: Firstly CGP78850 completely abolishes chemotaxis which would mean if transactivation occurs, than CCR5 mediated chemotaxis is completely signalling through transactivated RTKs. Whilst this seemed unlikely it is not impossible. The fact that inhibition of PKC ζ , which has been shown to be vital for RTK mediated chemotaxis in THP-1 cells [235] is unable to affect CCL3 stimulated migration and the dependence of THP-1 migration on non-receptor tyrosine kinases provided strong support for little involvement of transactivation. To resolve the issue more clearly, the Ras inhibitor farnesylthiosalicylic acid was used to treat THP-1 cells. Like CGP78850, farnesylthiosalicylic acid is poorly membrane permeable, however, significant reductions in Ras mediated signalling can be achieved with higher treatment levels [272]. Farnesylthiosalicylic acid is not able to block THP-1 cell migration at concentrations where it did not have a toxic effect on cells. This demonstrates that CCR5 signalling to chemotaxis is Grb2 dependent which represents a unique observation.

It is important to validate the observation made in THP-1 cells in peripheral human tissue for the reason described in previous chapters. The chemokine CXCL11 was first used to stimulate THP-1 cells to determine if CXCR3 is expressed on these cells and capable of inducing chemotaxis. THP-1 showed no propensity to migrate towards CXCL11 above basal movement demonstrating that CXCR3 receptor signalling in activated PBLs represents a distinct receptor system for comparison with CCR5 mediated events in THP-1. Activated PBLs are also known to express CCR5, which was confirmed by immunofluorescence staining with HEK 1/85/7a antibody and also by the fact that activated PBLs migrated towards CCL3, although, as discussed this may represent CCR1 activation. Interestingly dynasore treatment of PBLs prior to chemotaxis assay towards 20 nM CXCL11 has no significant effect on migration towards CCL3 was completely abrogated by the same treatment. Although dynasore had no effect on PBL

migration towards CXCL11 dyngo-4a was able to significantly reduced migration towards this chemokine. There are two possible explanations for this observation: firstly dyngo-4a may have cytotoxic or non-specific effects on PBL migration or secondly dynamin GTPase function is more important to PBL migration than THP-1 cell migration. As dyngo-4a appears to affect HeLa.RC49 morphology after 24hr, but dynasore did not, non-specific cytotoxic effects of dyngo-4a need to be investigated further before conclusions can be made. Focusing on dynasore, this is an important result because it indicates that CCR5 mediated chemotaxis is dynamin dependent across cell types, so long as the cell is small and motile and that chemokine receptor signalling between receptor families can be different. The PI3k inhibitors AS605240 and LY294002 were also used to treat activated PBLs. PBLs show a higher sensitivity to LY294003 than THP-1 cells, which suggests that CXCL3 functions through classical signalling to chemotaxis. This is supported by the previous experimentation which showed activated PBLs are sensitive to gallein treatment (Chapter 4). AS605240 does not affect activated PLB migration. The inhibitor is well characterised [216], but the continued negative results from both THP-1 cells and activated PBLs raise question about its functionality. The induced reduction of PKB phosphorylation would need to be assessed in THP-1 cells and PBLs to ensure the inhibitor is functioning properly.

The treatment of PBLs with CGP78850 strongly support the idea that dynamin is not important in CXCR3 mediated migration in PBLs and that the effect of dyngo-4a are more likely to be a result of non-specific actions. CGP78850 treatment at 100 μ M has no significant effect on PBL migration, demonstrating that signalling to chemotaxis in the cells was not dependent of Grb2 as it was in CCR5 mediated THP-1 migration. The fact that farnesylthiosalicylic acid treatment is also unable to block migration was unsurprising but did support the idea that signalling to migration for activated CXCR3 receptors follows a linear chemokine receptor specific path.

6.5 Chapter Conclusions

This chapter represents a pharmacological investigation into the roles of endocytosis and cytoskeletal machinery in CCR5 mediated chemotaxis. As such some interesting and novel

conclusions can be drawn regarding how chemotaxis can be modulated by small molecule means. Most interesting are the differential roles of dynamin in CCR5 and CXCR3 mediated chemotaxis where, based on the treatment with dynasore, CCR5 mediated chemotaxis in small mobile cells is dynamin dependent, whilst CXCR3 mediated chemotaxis is not. In the introduction it is explained that dynamin is a protein with a large and diverse range of cellular interactions which would make proteomic based investigation a long and costly exercise. The broad but generally poorly characterised range of small molecule inhibitors for dynamin allows a quick and cost effective analysis of its function. The use of a range of dynamin inhibitors with different binding sites and inhibitory functions demonstrates that dynasore is particularly effective at inhibiting CCR5 mediated migration and this potency may be based around an interaction between the inhibitor and dynamin that are not GTPase dependent. Indeed the observed effects of dynamin inhibitors did not correlate to GTPase or receptor mediated internalisation potency or their ability to stabilise dynamin rings which suggests something unique about the structure of dynasore. If this is the case, then structure activity modification to dynasore based around inhibition of migration rather than GTPase turnover may reveal potential therapeutics which affect migration but not internalisation. Another point of interest is the potential for CCR5 chemotaxis to be reduced by inhibition of Grb2, a dynamin targeting adaptor protein. If the inhibitor effects of CGP78850 are a result of the targeting of dynamin to sites of tyrosine phosphorylation, then Grb2 may represent a novel anti-migratory target for CCR5 stimulated migratory diseases. Grb2 inhibition may represent a means of targeting the chemotactic functions of dynamin without affecting its other roles in endocytosis or mitotic progression, although these points remain to be investigated. Grb2 may also be a better target than the non-receptor tyrosine kinases inhibited by bosutinib as the potential unwanted interactions of this inhibitor include proteins involved with programmed cell death [79].

Along with the benefits of speed, cost and target validation there are obvious limitations to a predominantly pharmacological investigation. Although a potential mechanism for Grb2 mediated CCR5 signalling to chemotaxis has been proposed the protein interactions between any of the protein species in this may not be direct. The specifics of protein-protein interactions

will fall into the remit of proteomics studies which, by knock down and overexpression studies, will be able to clarify the interaction broadly outlined in this thesis. What can be said with confidence is that dynamin 2, Grb2 and non-receptor tyrosine kinases make up group of proteins with strong evidence in the literature for interaction and with corresponding, and pharmacologically accessible effects in THP-1 migration. This thesis has validated these proteins as targets suitable for proteomic investigation with solid evidence that their disruption can reduce migration downstream of CCR5. The questions left unanswered by this thesis such as do arrestins and dynamin interact directly, where are Grb2 SH2 domains targeted and how exactly does ROCK become involved with THP-1 migration remains to be answered. These questions, rather than being detrimental, represent a large amount of potential future work with direct relevance to potential future therapeutics. The broader aspects of the research presented in this thesis will be presented in the final discussion chapter.

6.5.1. Final conclusions

- 1) Endocytosis appears to be negatively associated with migration indicating arrestin dependent signalling.
- 2) PI3K activation is not required for CCR5 mediated migration in THP-1 cells.
- 3) Dynamin is involved with THP-1 migration but inhibition of dynamin GTPase function does not correlate with decreased migration.
- 4) The dynamin binding protein Grb2 is required for THP-1 migration and may represent a link between non-receptor tyrosine kinases and dynamin in THP-1 migration.

CHAPTER 7: Final discussion and thesis conclusions

7.1. Final discussion

In this thesis we have undertaken a pharmacological characterisation of CCL3 stimulated chemotaxis in THP-1 cell line with the aim of identifying potential cytoplasmic signal transduction proteins that can be targeted as therapies for CCR5 mediated chronic immune and metastatic disease. The overarching conclusions from this research is as follows:

1. CCL3 stimulated calcium release and migration in THP-1 cells occurs via CCR1 and CCR5 in approximately equal proportions. CCR1 antagonist J113863 and CCR5 antagonist maraviroc cannot be used to isolate these responses from one another due to non-specific or agonistic effects demonstrated by the inhibitors.
2. G β γ , ERK1/2, p38 and PI3K are not important in THP-1 cell migration stimulated by CCL3.
3. PKC and GRK2 inhibition affect CCL3 stimulated migration in THP-1 antagonistically. This suggests CCL3 stimulated migration through (CCR1 & CCR5) occurs via arrestin dependent signalling.
4. JAK/STAT signalling does not form part of the cytoplasmic signal transduction pathway linking activated CCR5 to migration.
5. Internalisation of clathrin or caveolin dependent means is not required for migration but inhibition of internalisation increases migration.
6. Dynamin inhibition completely abrogates CCL3 mediated migration in THP-1 and HeLa.RC49, but CXCL3 stimulated migration in PBLs is unaffected.
7. Dynamin inhibitors do not function equally: this may represent a potential avenue of SAR development. Some dynamin inhibitors also have non-specific effects on calcium mobilisation in THP-1 cells.
8. Grb2 and c-Src inhibition decreases CCL3 stimulated migration in a way not explained by receptor tyrosine kinase inhibition.

It is important to analyse these conclusions in context of the initial hypotheses stated in the introduction. This was to identify potential cytoplasmic points of access to CCR5 mediated chemotaxis to by-pass the problems with receptor redundancy and transactivation. There is a need for therapies which will block cell migration, since there is a major involvement of chemokine receptors in both chronic inflammatory and metastatic disease [277-279]. So far, chemokine receptor as therapeutic targets have not resulted in sufficiently efficacious therapies, which is probably due to the intrinsic problems of extracellular inhibition of chemokine receptors such as receptor-ligand redundancy [278]. This is exemplified by conclusion 1 which demonstrates both the redundancy in chemokine receptor induced cellular responses and the difficulties in antagonist blockade of chemokine receptors. It should be noted that in this particular instance the effect of the chemokine receptor antagonists was due to previously unreported effects; non-specific for J113863 and partial agonistic for maraviroc. These unreported effects clearly indicate the potential for rationally designed small molecule inhibitors to affect chemokine receptor signalling in unforeseen and ways adding to the complications of redundancy and receptor cross-talk. There is some evidence that the failure for the production of effective chemokine based therapies is not due to signalling redundancy but failure to select valid biological targets [277]. If this were the case this would still not eliminate the problems observed with J113863 and maraviroc and supports the idea that identifying intracellular targets specific to chemokine receptor signalling maybe a more elegant solution.

Resolving the intracellular signal transduction specific to CCR5 is not possible due to the problems with J113863 and maraviroc, however, CCL3 receptors CCR1 and CCR5 share high levels of sequence homology and resolution of signal transduction at the level of two receptors is still useful. CCL3 stimulated responses in THP-1 were not sensitive to G $\beta\gamma$, PI3k, ERK or p38 MAPK inhibition (conclusion 2). This provides strong evidence that G $\beta\gamma$ and Gai (see Figure 1.5) [96] are not involved in signalling to migration, supporting the observations from Cardaba *et al.* [75] where calcium release was also not required. These observations highlight the potential for inhibitors of well validated signalling targets such as PI3K [78, 109], ERK [58, 82], and G $\beta\gamma$ [57] to be ineffective against certain diseases where CCR1/5-like signalling occurs.

By process of elimination, arrestin dependent signalling appears to be responsible for CCL3 stimulated migration in THP-1 cells. The use of PKC and GRK2 inhibitors supports this assertion and indicates that the mechanism by which arrestin recruitment occurs has significant effects on signalling to chemotaxis (conclusion 3). Inhibition of internalisation increases migration (conclusion 5), supporting the role of arrestin dependent signalling in this system. Arrestin, GRK and PKC appear to be involved with migration, but their suitability as targets for anti-migration therapeutics is low due to their lack of ubiquitous involvement in GPCR signalling and the potential for inhibition to increase pathological signalling by preventing desensitisation. Identifying players in arrestin dependent signalling to migration is likely to offer higher validity targets.

JAK/STAT signalling is not an important player in the cytoplasmic signalling events associated with CCL3 stimulated migration in THP-1 cells (conclusion 4), which correlates with the observed lack of sensitivity to PI3K inhibition. Treatment with the dynamin inhibitor dynasore, however, reveals a potential role for dynamin in CCL3 stimulated migration (conclusion 6). Dynasore is not effective in blocking CXCR3 stimulated PBL migration but it blocks CCL3 stimulated migration in the same system, suggesting a receptor family specific point of access to migration. CXCR3 and CCR5 have different signalling and desensitisation with CXCR3 mediated T-cell chemotaxis occurring in a PTX and PLC dependent fashion [280]. This correlates with the observed effect of the G β y inhibitor gallein in activated PBLs and suggests that migration in these cells might also be calcium sensitive, unlike CCL3 stimulated migration in THP-1 cells. Interestingly, dynamin inhibitors vary widely in their ability to affect CCL3 stimulated migration in THP-1 cells. Cross analysis of different dynamin inhibitors revealed that dynamin GTPase, RME and dynamin ring stabilisation inhibitory efficacy does not correlate to anti-migratory efficacy.

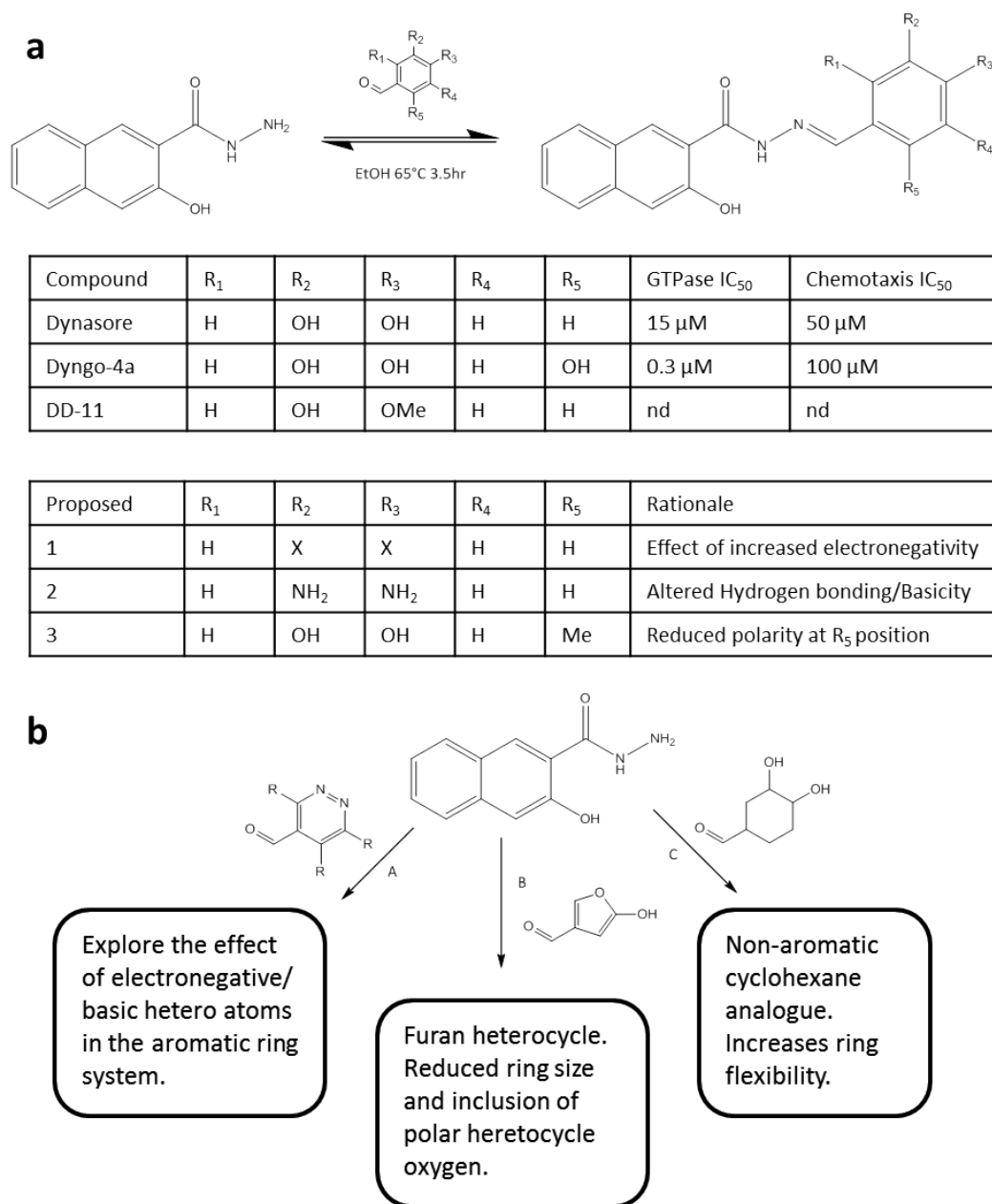


Figure 7.1: Basic synthetic strategy for SAR of migration specific dynamin inhibitors. (a) Existing analogues of dynasore, their structure and activity as GTPase and migration inhibitors. Compound DD-11 has been synthesised by Lee *et al.* and displays similar GTPase inhibition to dynasore but has not been tested in migration assays. Also shown are proposed structures to explore the relationship between migration inhibition and terminal aryl functional group. **(b)** Proposed ideas for structurally divergent migration specific dynamin inhibitors. Route 'A' explores heteroatom in place of hydroxyl moieties; route 'B' explored ring size and route 'C' explores the effect of increase ring flexibility. Reagents and conditions are conducive for fast, cheap production of a compound library.

The dynasore analogue dyngo-4a has a higher GTPase inhibitory potency than dynasore (Table 1.3) but is less potent as an inhibitor of migration. The fact that these compounds differ by one terminal aryl hydroxyl moiety and that this difference has such a profound effect on anti-migratory potency suggests that a SAR study to uncover potent inhibitors of migration that are poor inhibitors of dynamin GTPase function and therefore internalisation would be useful (Figure 7.1.).

Inhibition of dynamin that completely blocks endocytosis is likely to be detrimental to the normal functioning of cells and cellular processes; this thesis suggests that synthesis of 'migration specific' dynamin inhibitors maybe possible using chemotaxis assay as the test criteria. Figure 7.1a highlights dynasore analogues found in the literature: the differences between dynasore and dyngo-4a pivot on one terminal hydroxyl (Figure 7.1a), suggesting subtle modifications around this locus may yield significant changes in GTPase and migration inhibition efficacy. Compound DD-11, synthesised by Lee *et al.* [281] showed comparable RME efficacy to dynasore, indicating that the effect of modification at the terminal hydroxyls has some plasticity with regard to functional group type. This compound was not tested in GTPase or chemotaxis assay, so direct comparisons cannot be made. Due to the ease of synthesis and low cost of reagents, relatively divergent chemical species could be generated and tested with little effort (Figure 7.1b), allowing better understanding of the inhibitor-protein interaction to be gained quickly.

As dynasore and its analogue are the most effective inhibitors of migration and their hypothesised binding location is proximal to dynamin's regulatory PRD domain exploring PRD interacting proteins was an obvious step. Grb2 inhibitors block CCL3 mediated migration, the first time such a relationship has been established. Grb2 inhibition may represent a route to indirectly inhibit the migration specific functions of dynamin and showed some specificity towards CCL3 stimulated migration. Grb2 is known to target SH2 bound targets to SH3 domains activated by tyrosine kinases [112]; the c-Src inhibitor bosutinib was used to demonstrate that cytosolic non-receptor tyrosine kinases were vital for CCL3 mediated migration. Bosutinib is also

effective at reducing CXCR3 stimulated PBL migration, which raises question as to its specificity to any particular receptor signalling system. The use of bosutinib allows a comparative analysis of the efficacy of dynamin and Grb2 inhibitors against a drug that is soon to be released as an anti-cancer therapeutic [282]. Although their potencies are lower, both CGP78850 and dynasore are as effective at reducing migration as bosutinib, supporting the validity of Grb2 and dynamin inhibition as a potential therapeutic avenue for metastatic disease.

Table 7.1 lists the protein targets described in this thesis with regard to their validity as therapeutic targets against CCL3 (CCR1/CCR5) mediated chemotaxis. The blockade of the extracellular portions of the receptors themselves has not resulted in the production chemokine receptor antagonist based treatments for any disease. The difficulty in isolating CCR5 specific signalling from CCR1 specific events *in vitro* is exemplified in chapter 3, where both redundancy through lack of ligand specificity and non-specific antagonistic effects were observed. These problems are likely to be exacerbated *in vivo* where other factors such as increased extracellular ligand diversity are sure to reduce chemokine receptor antagonist efficacy. Taken as a whole, targeting chemokine receptors directly represents a poor target choice for anti-migratory therapeutics. Arrestin dependent signalling was identified as a likely route for signal transduction to migration in THP-1 cell upon CCL3 stimulation (chapter 4). Targeting arrestin directly does not represent a valid strategy for two reasons; firstly all GPCRs require arrestin for regulation. 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 where 'classical' G-protein mediated signalling was occurring which could have severe negative effects physiologically. Secondly there are presently no small molecule inhibitors of arrestin described adding a further element of complexity with regard to the *de novo* discovery of small molecule arrestin antagonists.

Table 7.1: Validity of proteins as targets in CCL3 stimulated chemotaxis.

Protein	Target validity	Rationale	References
CCR1/5	Very poor	Blockade of chemokine receptor signalling fails due signalling redundancy resulting from lack of receptor-ligand specificity as exemplified by the effects of maraviroc and J113863 on THP-1 cell chemotaxis.	Chapter 3,
Arrestin	Poor	Outside of arrestin dependent signalling, arrestin is required for desensitisation of all GPCRs therefore is likely to impact other GPCR signalling adversely. β arrestin isotype specificity would also need to be taken into account.	Chapter 4,
PI3K	Poor	Although validated as a therapeutic target for numerous cancers PI3K has an unclear role in CCL3 induced migration which may or may not be a result of cell specific signal transduction.	Chapter 6,
Dynamin 2	Moderate	Complete inhibition of dynamin would make a poor target for the blockade of chemokine receptor mediated migration as endocytosis is vital for normal cellular function. If migration-specific dynamin inhibitors can be developed however targeting the protein may be a realistic route for migration blockade.	Chapter 6,
c-Src	Good	c-Src inhibition is effective in THP-1 and HeLa cells and PBLs but may lack specificity between receptor types and has been linked to negative cellular responses.	Chapter 4,
Grb2	Very Good	Grb2 inhibition appears to affect CCL3 induced responses more than CXCR3 stimulated responses which may indicate specificity toward C-C motif ligand stimulated responses. Also has the potential to access RTK mediated signalling.	Chapter 6,

PI3K also represents a poor target in the CCL3-CCR5/CCR1 signalling axis, as its exact role could not be defined and sensitivity to PI3K inhibitors is lower than seen in systems where PI3K has been identified as a valid target [248]. The observed lack of efficacy of PI3K inhibitors (chapter 6) may have been a result of cell type specific responses however cell type diversity *in vivo* is high. This suggests that the efficacy of PI3K inhibitors *in vivo* may vary accordingly which is not ideal when dosing regimes and patient responsiveness need to be considered.

Dynamin may represent a valid target for inhibition provided that inhibitors which target migration specific functions can be produced. Failing this, dynamin does not represent a much better target than arrestin; systemic blockade of internalisation is likely to have severe negative physiological effects. The dynamin interacting protein Grb2 represents the most valid target described in this thesis; efficacy of Grb2 inhibition is comparable to the c-Src inhibitor bosutinib, which suggests with enhanced potency a Grb2 inhibitor may represent a point of pharmacological access to chemokine receptor mediated migration. The Grb2 inhibitor CGP78850 shows better efficacy in CCL3 mediated THP-1 migration than it did in CXCL11 stimulated PBL migration, which correlates with the observed effects of dynasore in these systems. This conclusion comes with the caveat that the interactions of Grb2 in chemokine receptor signalling are very poorly characterised; resolution of Grb2-Dynamin-chemokine receptor interaction proteomically will be important in validating this research.

7.2. Future work

As described in the introduction (chapter 1) the aim of this thesis is to resolve, by small molecule intervention, signal transduction from the chemokine receptor CCR5. This was achieved to a certain extent; although CCR5 specific events could not be isolated. Signalling stimulated by CCL3 via CCR1/5 was shown to occur through arrestin dependent signalling and to involve dynamin, Grb2 and c-Src. There are limitations to the conclusions that can be drawn from a predominantly small molecule based investigation; this was understood from the outset with the aim that the conclusions drawn from this thesis would allow more in-depth proteomic

characterisations to be undertaken. A relationship between dynamin inhibitors and anti-migratory efficacy was also discovered however, so the future work based on this thesis falls into four areas.

1. Further *in vitro* small molecule investigation to validate observations made in the thesis.
2. A proteomic investigation of Grb2 and dynamin and their interaction *in vitro*.
3. *In vitro* and *ex vivo* validation of the protein targets in disease models.
4. Synthesis of dynasore analogues to form SAR that allows 'migration specific' dynamin inhibitors to be produced.

Area 1 would involve experiments such as further small molecule validation of the identified protein targets. Obtaining or synthesising the pro-drug form of CGP78850 would allow the true potency of Grb2 inhibition to be determined and compared to clinically approved therapeutics such as bosutinib. A range of new dynamin inhibitors have recently been described based on dynole 34-2 [283]; testing these inhibitors may validate the hypothesised relationship between inhibitor binding and anti-migratory efficacy. Area 2 would involve basic proteomics such as the effect of Grb2 knock down and overexpression on migration and the effect of siRNA on dynamin interacting proteins which do not have small molecule inhibitors such as cortactin and gridin. More complex proteomics such as the production of PRD modified dynamin for overexpression or production of monovalent Grb2 might outline the exact functions of these proteins. Development of methodology to allow reliable transfection of THP-1 cells which does not impact migration assay is also an important point for future research.

Area 3 will validate the effect of the identified proteins in disease models. Animal models are the obvious choice but *ex vivo* studies such as monocyte migration towards rheumatoid arthritis synovial fluid offer a less costly way to validate the likely anti-migratory effects of Grb2 and dynamin inhibition *in vivo*.

Finally the design and synthesis of dynasore analogues with the structure activity relationship geared towards migration instead of dynamin GTPase function may allow novel small molecules that are poor inhibitors of internalisation but potent inhibitors of chemotaxis to be described. The simplicity of the synthetic route and low cost of synthetic reagents means that a diverse library of potential compounds could be produced and tested quickly. As a whole this future work, in addition to the research presented in the thesis, has the potential to enhance productivity an area of pharmacology that has historically underachieved in light of its therapeutic potential.

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