The Role of Microvesicles in Cancer and Viral Infection

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Abstract

Microvesicles are shed constitutively, or upon activation from both normal and malignant cells. Although recent studies have reported various nonlytic virus release mechanisms, this mode of virus transmission to secondary sites of infection has remained unclear. This study identified that Coxsackie virus B1 (CVB1) entry into HeLa cells results in apoptosis and production of virus-induced apoptotic microvesicles (vaMVs) by infected cells. Flow cytometery and fluorescence microscopy data illustrated that these vaMVs carry and disseminate CVB1 virions to new host cells via a nonlytic MV-to-cell viral mechanism. Inhibition of MV production by siRNA knockdown of *CAPNS1* in HeLa cells suggested that these vesicles mediate the spread of apoptosis to secondary sites of infection and the vaMVs could mediate nonlytic MV-to-cell transmission. This thesis also identified a new mechanism for multi-drug resistance involving the efflux of anticancer drugs from cancer cells mediated by release of microvesicles, removing the drug from treated cancer cells. Immunoblotting and flow cytometery data showed that transcriptional silencing of calpain by siRNA knockdown of *CAPNS1* in PC3M cells prior to drug treatment inhibits MV release and results in induced apoptosis in cells. This mechanism contributes to understanding the reasons for insensitivity to drug-induced apoptosis and the induction of drug-detoxification by cancer cells. This study has yielded important information about how to circumvent drug resistance to improve cancer chemotherapy. Furthermore, fluorescence microscopy results postulate that induction of MV release with agonist agents and anticancer drugs, results in damage to the host plasma membrane, which must be resealed immediately using activated lysosomes if the host cell is to survive and proliferate.

Original Publications

This report is based on the following published articles, abstracts and public presentations. In addition some unpublished data are presented.

I. Jorfi,S., and Inal, J.I (2013). Coxsackievirus B transmission and possible new roles for extracellular vesicles. *Biochem Soc Trans.* 1;41(1):299-302. doi: 10.1042/BST20120272.

II. Jorfi,S., and Inal, J.I (2013). The role of microvesicles in cancer progression and drug resistance, *Biochem Soc Trans.* 1;41(1):293-8. doi: 10.1042/BST20120273.

III. Jorfi, S., Ansa-Addo, E.A. and Inal, J.I (2012). Role of Microvesicles in cancer drug resistance and membrane resealing. (*Manuscript in preparation*).

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V. Grant, R., Ansa-Addo, E., Stratton, D., Antwi-Baffour, S., Jorfi, S., Kholia, S., Krige, L., Lange, S., INAL, J.M. (2011) A filtration-based protocol to isolate Plasma Membrane-derived Vesicles and exosomes from blood plasma *J. Immunol Methods. 371,143.*

VI. Inal, J.M., Ansa-Addo, E., Stratton, D., Kholia, S., Antwi-Baffour, S., Jorfi, S. and Lange, S. (2010) Microvesicles in health and disease. Arch. *Immunol., Ther. Exp. 60.*

Published Abstracts

VII. Jorfi, S., and Inal, J.I (2012) Cancer cell expulsion of anticancer drugs through shedding of microvesicles: Association with drug resistance and tumour survival. *Biochemical Society Transactions.*

VIII. Jorfi, S., **Ansa-Addo, E.A. and Inal, J.I (2012)** Coxsackie virus entry and spread in HeLa cells is aided by microvesicle release. *J Immunol* **188**:170.22

IX. Jorfi, S., **Ansa-Addo, E.A. and Inal, J.I (2010).** Plasma Membrane-derived Vesicles derived from apoptotic Jurkat cell express upregulated surface Fas and induce apoptosis in recipient viable cells. *J Immunol* **184.**

X. Jorfi, S., **Ansa-Addo, E., and Inal, J.M (2010)** Plasma Membrane-derived Vesicles released from infected HeLa cells by Coxsackie virus B1 induce apoptosis in recipient viable cells. *J Immunol* ***184:89.45.***

Public Presentations

XI. Jorfi, S., **and Inal, J.M (2012)** The role of microvesiculation in determining the sensitivity of cancer cells to chemotherapeutic drugs. Presented at Microvesiculation and disease 13th-14th September 2012 London Metropolitan University, UK. Sponsored by Biochemical Society-awarded first prize for the best poster.

XII. Jorfi, S., **and Inal, J.M (2012)** Role of Microvesicles in cancer drug resistance and tumour survival. Presented at the 3rd Postgraduate Research Students Symposium held at London Metropolitan University on 12th July 2012 - awarded first prize for the best presentation.

XIII. Jorfi, S., **Ansa-Addo, E., and Inal, J.M (2010)** Plasma Membrane-derived Vesicles derived from apoptotic Jurkat cell express upregulated surface Fas and induce apoptosis in recipient viable cells. Presented at the 97th Annual Meeting of the American Association of Immunologists (AAI) from 7-11th May 2010.

Abbreviations

ATP	Adenosine triphosphate
An V	Annexin V
aMV	Apoptotic Microvesicle
BSA	Bovine serum albumin
BzATP	3'-*O*-(4-benzoylbenzoyl)-ATP
Cal-I	calcium ionophore
CAPNS1 *siRNA*	Calpain small-subunit 1 small interfering RNA
CP	Calpeptin
$[Ca^{2}+]i$	Intracellular calcium
CD4	Cluster of differentiation 4
CHX	Cychloheximide
ConA	Concanavalin A
Crtl	Control
CVB1	Cosxackie Virus B1
CytD	Cytochalasin D
DAF	Decay accelerating factor
DDSA	Dodecenyl succinic anhydride
DEAE-cellulose	Diethyaminoethyl cellulose
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
Doc	Docetoxel
DTT	Dithio threitol
ECM	Extracellular matrix
ECS	Extracellular space
EGTA	Ethylene glycol-bis (2-aminoethylether) N,N,N',N'-tetraacetic acid
ELISA	Enzyme Linked immunosorbent assay
ER	Endoplasmic reticulum
Exos	Exosomes
FACS	Fluorescent activated cell sorter
FasL	Fas ligand
FasR	Fas receptor
FGF-1	Fibroblast growth factor -1
FSC	Forward scatter
g	G-force
Gal-3	Galectin-3
G-CSF	Granulocyte - colony stimulating factor
GsMTx-4	Grammostola spatulata mechanotoxin4
HIV-1	Human immunodeficiency virus 1
HPP	HiPerfect transfection reagent
Hsp	Heat-shock protein
HUVECs	Human umbilical vein endothelial cells
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-12	Interleukin 12
5-FU	5-Fluorouracil

LAMP-1	Lysosome associated membrane protein1
MAC	Membrane attack complex
MHC	Major histocompatibility complex
MPs	Microparticles
mRNA	messenger Ribonucleic acid
MSC	Mechanosensitive channels
MTX	Metothroxate
M-tropic	Macrophage-tropic
MVs	Microvesicles
MVBs	Multivesicular bodies
NI	Non-Induced
PB	Peripheral blood
PBB	Permeabilisation buffer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline – Tween
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEAM	Platelet expressed adhesion molecules
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidyl inositol-3-kinases
PL	Phospholipid
PM	Plasma membrane
PMN	Polymorphonuclear neutrophils
PMSF	Phenylmethanesulfonyl Fluoride
PMVs	Plasma membrane-derived vesicles
PMP	Platelet microparticles
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
RBCs	Red Blood Cells
RNA	Ribonucleic acid
RT	Room temperature
SAC	Stretch activated calcium channel
SD	Standard deviation
SSC	Side scatter
SM	Sphingomyelin
TEMED	N, N, N-tetramethylethylenediamine
TF	Tissue factor
TGF-β1	Transforming growth factor β 1
TTP	Thrombocytopenic purpura
TPA	Tissue plasminogen activator
v/v	Volume per volume
vaMVs	CVB1 stimulates apoptotic MVs
w/v	Weight per volume
ZVKD-fmk	pan-caspase inhibitor

1. Introduction

1. Introduction

1.1 Microvesicles

For a long time, cell communication has been defined as the direct secretion and interaction of growth factors, small molecular mediators, enzymes, cytokines and gradient of soluble ligands. However, recent findings describe another mode of cell-to-cell communication that has probably existed throughout evolutionary time, involving the release of small cellular fragments termed microvesicles (MVs) [1, 2].

Multicellular organisms are subjected to a wide range of stimulants during each stage of their development. During homeostasis, a balance between cell proliferation and degeneration controls the cell cycle, allowing cells to differentiate and function, and subsequently undergo apoptosis that ends with ingestion and digestion by phagocytes, termed phagocytosis. At every stage, cells are challenged and stressed leading to the release of small membrane-coated vesicles termed microvesicles (MVs) or microparticles (MPs)[3]. These submicron vesicles carry cytosolic components and membrane constituents similar to the parental cell. MVs of the same cellular origin may have different protein and lipid compositions[4].

Microvesicles (MVs) or Microparticles (MPs) are small, intact heterogeneous membrane vesicles (with a diameter of 0.1- ≤1μm) that contain elements involved in cell signalling and intercellular communication[5]. A variety of cell types including platelets, neutrophils, reticulocytes, macrophages,

megakaryocytes, monocytes, B and T cells, mast cells, endothelial cells, and tumour cell lines have all been described to release MVs either constitutively or upon stimulation with an extracellular stimulus[6-8]. MVs also vary in size, composition and biological effects. The process of microvesiculation allows a selective release of cell components to the surrounding microenvironment[5]. Moreover, chemical and physical factors in the eukaryotic cell environment elicit microvesicle release during cell activation, stress, apoptosis, malignant transformation, hypoxia and differentiation[9, 10]. MVs also engulf some cytoplasm during shedding through their membrane and as a result contain proteins, microRNA and mRNA derived from parental cell[11].

In addition, MVs have also been reported to carry infectious particles (HIV or prions) and organelles such as mitochondria[12]. As they differ slightly in their composition depending on status and cell origin, MVs are referred to by various terms in a number of reports. For example, monocyte and platelet-derived MVs are referred to as microparticles[13, 14]. Moreover MVs derived from the basolateral membrane of cells that synthesize morphogens[15] during tissue development are called argosomes[16] and those produced by activated human polymorphonuclear neutrophils (PMN) are referred to in the literature as ectosomes[17]. In this thesis, microvesicles and not exosomes will be referred to as MVs.

1.2 Definition and historical background

MVs can simply be described as small heterogeneous vesicles released from the surface membrane of most cells. They are subcellular fragments which participate in intercellular communication and cell signalling upon release[1, 5]. They vary in sizes, ranging from approximately 0.1 to 1 μm in diameter, and have different compositions and biological effects[18]. Upon release, these MVs are rich in actin and have phosphatidylserine (PS) exposed on the outer leaflet of their membrane. PS externalization can be detected through binding assays using a fluorescence conjugated annexin V a well-accepted method of detecting and quantifying MVs by flow cytometry[19, 20].

MVs were first reported in 1949 when Chargaff et al. recognised a precipitable factor that accelerated the generation of thrombin was present in platelet-free plasma[21]. Using ultracentrifugation, platelet-derived fragments were successfully isolated from the platelet-free plasma, and these fragments were capable of producing thrombin. Moreover, a direct correlation was reported between the amounts of platelet microparticles (PMP), referred to as "platelet dust", and the platelet count present in the original blood sample[22]. Consistently, there were higher levels of PMP in polycythaemic patients with higher platelet counts, but lower levels in thrombocytopenic patients[22]. This implied a role for PMP in blood clotting.

An increase in intracellular calcium levels culminates in rearrangement of the actin cytoskeleton and leads to the release of MVs from the host plasma

membrane (PM) by exocytic budding[23]. MVs play important roles in crosstalk communication and they transfer and regulate processes such as apoptosis, inflammation, coagulation and proliferation between cells. As a result, MVs have been well documented to play important roles in various diseases[8, 24].

1.3 Mechanism of formation

1.3.1 Physiological and exogenous stimuli induce MV release

Microvesiculation is a tightly regulated process with multiple mechanisms including physiological and exogenous agents involved in the release of MVs. Physiological agonists such as sublytic concentrations of the complement membrane attack complex (C5b-9)[25] or exogenous stimuli such as epinephrine, ADP, collagen, thrombin and calcium ionophore can be used to stimulate production of MVs *in vitro*[26].

Under steady state conditions, the cell membrane is asymmetric in relation to the composition and the distribution of phospholipids in its inner and outer layers: phosphatidylcholine and sphingomyelin are located in the outer layer, while PS and phosphatidyl-ethanolamine (PE) are present in the inner leaflet[27]. Under normal physiological conditions, most eukaryotic cells have an asymmetric distribution of membrane phospholipids. This plasma membrane asymmetry is regulated by transmembrane enzymes, which promote transverse migration of anionic phospholipids such as PS from the inner to outer leaflet of the plasma membrane prior to the release of MVs[10, 28, 29]. Any cellular events that lead to the loss of cell asymmetry mostly result in

MV release. The cytoskeleton is therefore disrupted, resulting in membrane budding and microvesicles shedding[27, 29].

MV release during cell activation, injury, apoptosis and necrosis may lead to the loss of cell asymmetry and exposure of PS on the outer membrane leaflet. In blood platelets, PS exposure on the outer layer of the cell membrane may in turn result in a prothrombotic state[30, 31]; however, PS exposure also promotes clearance of apoptotic cells and cell fragments by macrophages[32].

1.3.2 Enzymes involved in governing plasma membrane asymmetry and MV release

MV release is a calcium-dependent process, and as such could be inhibited by calcium chelating agents such as EGTA and EDTA. Five enzymes are involved in regulating the asymmetry of the plasma membrane: floppase, scramblase, calpain, gelsolin (only in platelets) and aminophospholipid translocase[10]. In the resting state, PE and PS are predominantly expressed in the inner layer of the plasma membrane. By contrast, phosphatidylcholine (PC) and sphingomyelin are enriched in the outer membrane. Calcium is mainly stored in the endoplasmic reticulum (ER)[33] (**Figure 1.1**). In the resting state, scramblase and floppase, an ATP-dependent enzyme that contributes to maintaining the physiological asymmetry of the membrane remain inactive. By contrast, flipase, an ATP-dependent translocase is active and transports PS and PE from the outer leaflet of the PM to the inner layer, thus maintaining PS and PE inside[34]. Under this condition, choline phospholipids

are expressed on the outer leaflet, whilst aminophospholipids are exposed in the inner layer of the PM[35]. The influx of calcium leads to stimulation of scramblase and floppase and concomitant inhibition of flipase[36].

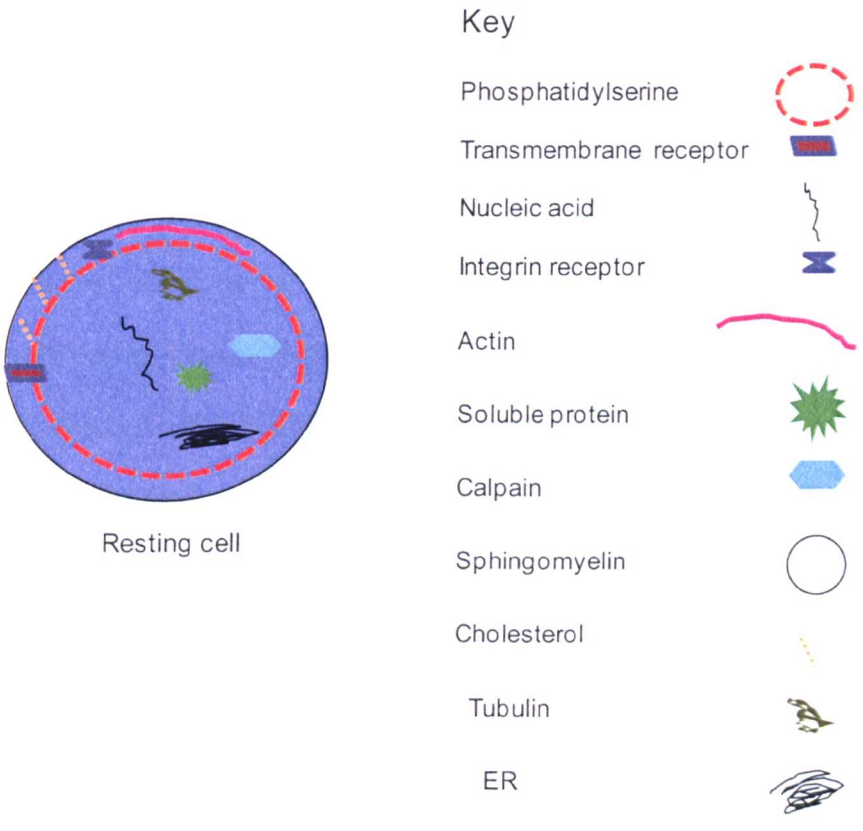


Figure 1.1 Schematic representation of a resting cell cytoskeleton.
In resting state calcium is stored in the endoplasmic reticulum (ER). Phosphatidylserine and phosphatidyl-ethanolamine are maintained in the inner leaflet of plasma membrane.

1.3.2.1 Gelsolin

Gelsolin is activated by the influx of Ca^{2+} which leads to release of MVs in platelets. Once activated, this enzyme removes capping proteins located at the ends of the actin filaments in the platelet cytoskeleton, thus leading to rearrangement of the long actin filaments causing the cell to contract. This in turn causes the actin filaments to reorganize and hence causes platelet activation, contraction and subsequent release of MVs[37].

1.3.2.2 Aminophospholipid translocase

An inward-directed pump, aminophospholipid translocase or flipase [Mg^{2+}- and adenosine triphosphate (ATP)-dependent enzyme] controls the strict translocation of PS and PE against an electrochemical gradient, from the outer leaflet to the inner layer of the cell membrane at resting state. For each PS molecule transported, one molecule of ATP is required; but the action of flipase is inhibited by increases in intracellular calcium concentrations[38].

1.3.2.3 Floppase

Floppase is less dependent on Mg^{2+}/ATP and transports choline phospholipids (PC and sphingomyelin) from the inner layer to the outer layer of the plasma membrane; it is also activated by increases in intracellular calcium. Therefore, in resting state the membrane asymmetry is controlled by flippase and floppase[33].

1.3.2.4 Scramblase

Scramblase is also activated by an increase in intracellular calcium levels, leading to budding of the PM. Together these events lead to changes in stability of the cell membrane and in cell conformation. As PS is externalized to the outer leaflet of the PM upon cell activation, MVs are hence characteristically released expressing PS on their outer membrane[39].

The rare autosomal recessive disorder of platelet coagulant activity, Scott's syndrome, has a high concentration of scramblase present in platelet plasma membrane; however due to an inherited defect, scramblase is unable to generate normal activity resulting in a reduced exposure of procoagulant PS on the surface membrane of the cell and also a reduction in MV production. In this condition the ability to activate factor X and prothrombin is impaired and thus the disorder is associated with severe bleeding[40, 41].

1.3.2.5 Calpain

Calpain (CP or Calp), a cysteine protease of the papainase family, is activated by elevated levels of calcium ions from extracellular sources and Ca^{2+} released by the endoplasmic reticulum (ER). Members of the calpain family can be classified into two subfamilies: μ-calpains (or calpain-1, are activated by micromolar concentrations of Ca^{2+}) and m-calpains (or calpain-2, are activated by millimolar concentrations of Ca^{2+})[23, 42]. Once activated, calpain translocates from the cytosol to intracellular membranes where they cleave a number of substrates including cytoskeletal proteins, adhesion

molecules, membrane proteins, kinases, phosphatases, ion transporters and phospholipases. Its functions vary but most importantly calpain cleaves the cytoskeletal actin filaments resulting in the production of MVs (**Figure 1.2**). Other functions include activating apoptosis through procaspase 3 and BclxL[42, 43].

To confirm involvement of calpain in microvesicle release, calpeptin, a known inhibitor of calpain was shown to inhibit the release of MVs from platelets[44]. The release of platelet MVs is strongly associated with the presence of circulating calpain activity in the plasma in thrombotic thrombocytopenic purpura (TTP)[43]. Calpain activation activates tubulin, and gelsolin cleaves the actin capping proteins leading to rearrangement of the long actin filaments[44]. Ca^{2+} influx also leads to activation of scramblase and floppase and deactivation of flipase by which point the asymmetry of phospholipids becomes compromised. Cleavage of the actin cytoskeleton leads to loss of cell asymmetry, followed by outward budding of the microvesicles from the plasma membrane[45]. Depending on the stimulus, specific proteins are localised within or on the surface of the budding vesicles before complete detachment from the original cell. Once released, MVs exhibit protein profiling similar to the parent cell and some cleaved actin is inside the vesicles. The plasma membrane of the released vesicle also inverts so that PS normally expressed on the inner leaflet of the MVs membrane would be exposed on the outer leaflet[19, 46, 47].

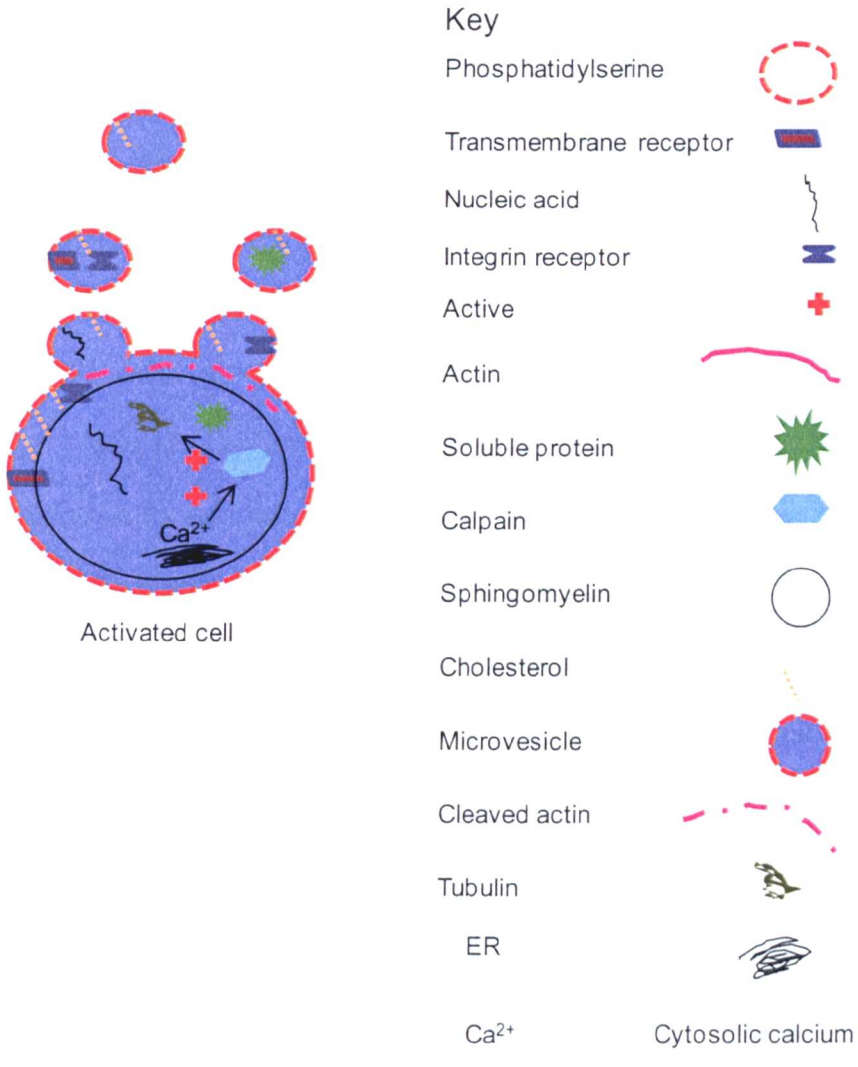


Figure 1.2 Initial responses following cell activation.
Increase in cytosolic calcium ions leads to the activation of calpain. Calpain activates another enzyme tubulin leading to rearrangement of the long actin filaments. The increased cytoplasmic Ca^{2+} also leads to activation of scramblase and floppase and deactivation of flipase, by which point the asymmetry of phospholipids starts to become compromised.

1.3.2.6 Lipid rafts

It is believed that MVs are released from lipid raft-rich regions of the cell membrane and this explains their observed enrichment in MVs[48]. Lipid rafts are enriched in certain lipid cholesterols and proteins, and can be defined as an ordered region floating in a sea of poorly arranged lipids. There are three types of lipid rafts: caveolae-specialised lipid rafts involved in signalling functions, (glyco) sphingolipid-enriched membranes (GEM), and polyphosphoinositol rich rafts (PIP2). They are also classified into inside rafts (PIP2 rich and caveolae) and outside rafts (GEM)[48, 49]. Sphingolipids consist of long, saturated acyl chains but phospholipids contain unsaturated acyl chains. Cholesterol preferentially partitions with sphingolipid bilayers, and occupies the space between the acyl chains. Cholesterol plays a crucial role in the stability of lipid rafts, because rafts have specific interplays with the cytoskeleton and participate in constitutive signalling[49, 50]. It has been postulated that lipid rafts may be shed in the form of MVs from the plasma membrane. This was due to lipid raft observations in several MV-like phenotypes released from endothelial cells and other types of cells after activation with different stimuli[51]. The idea that MVs are released from lipid raft domains was further suggested in a report, which showed that clustering of platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) preceded the shedding of MVs enriched in this molecule on the cell surface[52]. Because cholesterol is essential for the maintenance of the two membrane phases, extraction of cholesterol from the membrane results in the dispersal of lipid rafts. Therefore, cholesterol has been postulated as the dynamic glue that

holds lipid rafts together[52]. Further elucidation of the involvement of lipid rafts and other microdomains will enhance our understanding of the mechanisms, which lead to the release of MVs.

1.4 Apoptotic bodies differ from MVs

Apoptosis can be defined as a coordinated cell death program employed by multicellular organisms, which occurs after sufficient cellular damage and plays a pivotal role in many physiological processes. This damage brings about changes that involve fragmentation of the nucleus, cytoplasm and eventual blebbing of the plasma membrane. As a result, cells round up (in the process losing contact with neighbouring cells) and shrink during early stages of apoptosis[53].

In addition, nuclear chromatin starts to condense and aggregate into dense compact masses, which are degraded by endonucleases in the nucleus[53]. Flipase is deactivated and exposure of PS on the outer leaflet of the plasma membrane correlates with activation of scramblase, thus resulting in the release of MVs[54]. However, it was suggested that exposure of PS does not always lead to MV release. This is because the threshold of cytosolic Ca^{2+} required for MV formation is higher than for exposure of PS at the outer leaflet[55]. For example, MV release was not induced in the executive phase of apoptosis. Moreover, release of PS^{+}MVs by the topoisomerase I inhibitor, camptothecin (CPT) was further enhanced by inhibition of myosin adenosine triphosphatase (MAT). Simply, CPT-induced release of MVs was not inhibited by the myosin light chain inhibitor. Interestingly, a marked increase in the

release of PS^{+}MVs was observed with staurosporin (a general kinase inhibitor)[56].

These findings contradict previous reports, which postulated that apoptotic blebbing is dependent on the activities of myosin adenosine triphosphatase and myosin light chain kinase[56]. However, it indicates that PS+MVs released during the early stages of proapoptotic stimulation are produced via a different mechanism than apoptotic blebbing, which result in the release of apoptotic bodies[56]. Nusbaum et al (2004), initially set about to investigate the mechanism involved in downregulating leukosialin (CD43) during neutrophil apoptosis. However, they noticed that CD43 had previously been reported as the main sialoglycoprotein of the leukocyte plasma membrane with both adhesive and anti-adhesive properties[57, 58]. Another group suggested that CD43 expression levels decrease during neutrophil activation and adhesion due to proteolytic cleavage[57]. In view of this, they investigated the release of microvesicles during neutrophil apoptosis, in relation to the decreased expression of CD43. They found that decrease of CD43 was due to its release via MVs[57].

The extrinsic pathway of the caspases cascade and the intrinsic pathway of mitochondria cytochrome C release, and apoptosome formation are the signalling pathways for apoptosis. Although both pathways exist in neutrophils, use of the pan-caspase inhibitor, Z-VAD-fmk and the specific caspase-3 inhibitor, Z-DEVD-fmk, failed to inhibit PMN membrane blebbing,

albeit it down-regulated CD43 expression. It was concluded that PS-expression, membrane blebbing and CD43 decreased expression are distinct signalling mechanisms, which occur independently of each other[58].

1.5 Exosomes definition and functions

In addition to MVs, eukaryotic cells also release another type of vesicle called exosomes. This mechanism first was described in 1970s by Rose Johnstone [59]. Exosomes are smaller than MVs, ranging in size from 30-100 nm in diameter and are coated with a lipid bilayer and a density ranging from 1.13 g ml^{-1} (for B-cell derived exosomes) to 1.19 g ml^{-1} (for intestinal-cell-derived exosomes)[60]. Exosomes also carry protein biomarkers and express certain surface receptors identical to parental cells. A neutral sphingomyelinase (Smase) enzyme is the key controller of exosome biogenesis. Ceramide is produced by activation of neutral Smase which in turn hydrolyses sphingomyelinase on the cytosolic leaflet of the endosomal membrane creating a curvature resulting in an inward budding into the endosome. The intraluminal vesicles resulting from the endosomes are contained inside multivesicular bodies (MVB) and are called exosomes upon their release when the MVB fuses with the PM[61].

Exosomes play a significant role in antigen presentation and mediating death in target cells[62]. Specific proteins are considered in order to distinguish between exosomes and other vesicles. These are referred to as exosomal markers and found in all exosomes originated from various cell types. Among

these major histocompatibility complex (MHC) class I and II molecules and tetraspanins – including CD9, CD63, CD81 and CD82 represent the most abundant protein families present in exosomes[63].

Tetraspanins consist of a group of proteins with four transmembrane domains which are distinguished by a number of conserved amino acids in the transmembrane domain[64]. In addition, they have two different sizes of extracellular domains with yet unknown functions but are postulated to interact with integrins and MCH molecules. Exosomes are comprised of proteins such as tubulin, actin, actin-binding proteins (in cytoskeleton), annexin, and Rab proteins (involved in intracellular membrane fusion and transport) and many others[24, 60, 64, 65].

Numerous molecules involved in signal transduction including protein kinases and heterotrimeric G-proteins, various metabolic enzymes (e.g. peroxidases, pyruvate, lipid kinases and enolase-1) and heat-shock proteins such as Hsp70 and Hsp90 have been reported in exosomes. The latter are ubiquitous chaperone proteins, which are involved in antigen presentation, since they bind to antigenic peptides and participate in peptide loading onto MHC molecules[63, 64].

1.6 Methods of detection and quantification of MVs

1.6.1 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA and electron microscopy techniques are some of the methods used for detecting and distinguishing exosomes from MVs. In the ELISA method antibodies against MV antigens are used to identify MVs. This method employs a plate coated with annexin V, a calcium-dependent phospholipid binding protein naturally present in cells with a strong affinity for phosphatidylserine. It is also used in the presence of Ca^{2+} to detect PS+MVs by flow cytometry. ELISA is cost effective, user-friendly and allows the clinical diagnostic/screening of large number of samples. However, this technique also lacks direct quantification of MVs and does not provide information about the size and granulity of MVs[66]. In this study all MVs presented as PS+MVs were detected by fluorophore-conjugated annexin V.

1.6.2 Flow cytometry

Several methods for analysis of MVs have been reported, however flow cytometry and ELISA are the most commonly adopted. Here, it is impossible to make a direct comparison between results gained by different approaches such as centrifugation, re-suspension and washing of the MV pellet. Flow cytometry is the most common method of choice for analysing and studying MVs. It allows analysis of large numbers of MVs, up to tens of thousands and provides information about their corpuscular characteristics, forward scatter parameter correlating with the size of MVs and side scatter reflecting their granularity.

Flow cytometry also allows antibodies conjugated to three or even more antigens can be analysed on isolated MVs to be analysed. Similarly, accessibility of PS on the surface of MVs can be detected using annexin V conjugated to fluorophores such as FITC (fluorescein isothiocyanate) green in colour or PE (phycoerythrin) an energy transfer red dye. Samples are usually obtained from platelet-free plasma, other body fluids (urine, synovial fluid) and cell conditioned medium. Using labelled antibodies against cell-specific antigens or activation markers and annexin V, a protein that binds specifically to negatively charged phospholipids in the presence of calcium ions, MV fractions or subpopulations can be quantified and concurrently their cellular origin as well as their activation status established. Of each event detected by flow cytometry, the size (forward scatter-FSC) and density or granularity (side scatter-SSC) are determined electronically, as well as the fluorescence in various channels.

Fluorescence reflects the amount of antibody bound and therefore estimate the amount of antigen exposed on the membrane surface. The main disadvantage with most commercially available flow cytometers is that they fail to accurately analyse smaller MVs, which may be approximately 10-30 nm in diameter.

Interestingly, most groups analyse MVs using the forward scatter linear (FSC-H-Linear) and side scatter linear (SSC-H-linear) parameters on the flow cytometer[67]. However, we noticed using the Guava EasyCyte flow cytometer

(Guava Technologies, UK) that with these settings only a small fraction of the samples are actually analysed. When set to FSC-HLog and SSC-HLog, all the samples are displayed and so can be analysed accurately and this further highlights the advantage of the Guava EasyCyte, which is user-friendly, has a higher resolution and easier to maintain.

1.7 Physiologic and pathogenic activities of MVs

The study on MVs in health and disease has expanded our understanding about microvesiculation and helped abolish any doubts initially thought. This explains how MVs considered as disseminated storage pool of bioactive effectors are interpreted in intercellular communication and participate in the maintenance of homeostasis under physiological conditions. They initiate a deletion mechanism to remove excess molecules and also play significant roles in many physiological and pathophysiology processes including homeostasis, thrombosis, inflammation, modulation of vascular tone, angiogenesis, stem cell engraftment, tumour metastasis and others[9, 18, 68, 69].

1.7.1 Microvesicles in intercellular communication

Many reports point to MV-induced intercellular cross talk and show these vesicles to contain numerous proteins and lipids similar to those present on the plasma membranes of parent cells. For several years this mechanism has been shown as a way of cell interaction. Recent studies confirmed that release of MVs is associated with transfer of antigens and receptors to initiate cell signaling[1, 5, 70].

MVs induce cell-to-cell communication by direct cell stimulation, resulting in activation of a complex of signalling and transfer of surface receptors to local cells or delivering of proteins and mRNA to target cells[11]. It is well documented that cells release MVs in order to exchange information among themselves through various mechanisms such as release of growth factors, cytokines, chemokines and small molecular mediators such as nucleotides, nitric oxide ions and bioactive lipids[18, 71]. For instance, Leukocyte MVs activate endothelial cells by transferring leukocyte antigens to epithelial cells. This passive acquisition of leukocytic phenotypes is associated with changes in phosphorylation of cellular proteins and cell-cell adhesion properties[72]. Platelet-derived MVs modulate monocyte-endothelial cell interactions and stimulate proliferation, survival, adhesion, chemotaxis of hematopoietic cells, and also enhance engraftment of hematopoietic progenitor cells[66].

1.7.1.1 MVs are associated with signal transduction

Recent evidence in proteomic studies suggests that MVs carrying growth factors, chemokines and proteins are involved in cell signalling between different cell lines. MVs act as a signalling complex with crucial roles in cell communication. In addition, MVs derived from platelets are well documented to activate certain signalling pathways in human cells[73]. In both normal and malignant human haemopoietic cells phosphorylation of MAPKp42/44 and PI-3K-AKT signalling pathways is an effect platelet-derived MVs[73]. The activation of MAPKp42/44 and AKT further confirms that MVs interact with target cells and activate several signalling pathways which results in cell

proliferation and survival[74, 75]. MVs bearing Fas ligand can also be shed from the cell surface providing a mechanism for long-range signal directed apoptosis[76]. MVs derived from the basolateral membrane of cells bearing morphogens named argosomes, play essential roles in development and morphogenesis of multicellular organisms. These studies demonstrate that MVs can be efficient vectors of biological information from one cell type to another within proximal or remote tissues[15].

1.7.1.2 Transfer of membrane-associated receptors

In vitro studies showed that Platelet-derived MVs containing lipids components promote proliferation, migration, survival, and tube formation through a kinase dependent effect in target HUVECs. Moreover, the same MVs demonstrated proangiogenic activities both in *in vivo* and in *in vitro* studies[77]. In addition, chemokines receptor CCR5 is released from peripheral blood mononuclear cells (PBMCs) through MVs shedding. Concomitant incubation of these CCR5-bearing MVs with peripheral blood mononuclear cells (PBMCs) which are CCR5-negative resulted in detection of CCR5 receptor on PBMCs. This can aid binding of the macrophage-tropic HIV-1 to the surface of primarily non-susceptible cells and to subsequently facilitate internalization of viral particles[78].

Platelet-derived MVs express platelet-expressed adhesion molecules (PEAM) and transfer it to haemopoietic cells, resulting in an increase of their ability to adhere to fibrinogen or endothelium[76]. These reports were

supported by an earlier observation that platelet-derived MVs could transfer the CD41 antigen ($\alpha_{IIb}\beta_3$ integrin) to endothelial cells. They also transfer ($\alpha_{IIb}\beta_3$ integrin) to the tumour cells resulting in increased adhesiveness of lung cancer cells to endothelium and metastasis[69, 79].

Recently it was also shown that monocyte-derived MVs are not only enriched in P-selectin glycoprotein ligand-1 (PSGL-1), but also express high levels of tissue factor (TF). This fuses with activated platelets and initiates coagulation after circulating through the peripheral blood. As platelets themselves do not secrete TF, other mechanism(s) must be involved which help explain how they acquire this receptor during coagulation[48].

In previous works, it was demonstrated that if Jurkat T cells (CD81-positive) are co-cultured with U937 promonocytic cells (CD81-negative), U937 cells became positive for CD81, a tetraspan co-receptor involved in the activation of B and T cells. It was postulated that CD81 was carried and transferred by Jurkat cells-derived microvesicles to U937 cells. This CD81 positive U937 cells were now able to produce an immune response during infection showing that the receptor had retained its function[80].

MVs transfer receptors between cancer cells. Melanoma and ovarian cancer cells release Fas Ligand-bearing MVs (Fas-L). This ligand normally induces apoptosis in T cells, which express the Fas receptor (Fas-R); hence removal allows the tumour cells to escape immunosurveillance[81, 82].

A report confirmed that cells which do not typically express HIV-related co-receptors on their surface become susceptible to HIV infection. This can be explained by the idea that MVs released by cells, which are normally positive for these co-receptors can transfer the receptors to negative recipient cells. Moreover, a recent report showed that MVs derived from peripheral blood mononuclear cells (PBMCs) can transfer specific HIV-1 co-receptors onto endothelial cells during transendothelial migration, rendering these cells susceptible to HIV infection[83].

Platelet-derived MVs transfer CXCR4 receptor to CXCR4-null cells which results in susceptibility of these cells to HIV infection[84]. These studies showed that released MVs from these cells contain HIV-specific co-receptors and if these MVs are co-cultured with cells negative for the co-receptors rendering target cells susceptible to HIV infection[78, 85].

Furthermore, presented evidence confirmed the significance of release of MVs in association with ATP-binding cassette transporter A1 (*ABCA-1*), a receptor, which regulates distribution of PS at the outer leaflet of the plasma membrane during the release of MVs in cerebral malaria (CM)[86]. In *ABCA-1-/-* mice, MV release is reduced and leads to complete resistance to the development of cerebral malaria by these mice. Conversely, larger numbers of MVs were released in *ABCA-1+/+* mice and this resulted in development of CM when mice infected by the parasite *Plasmodium bergbei* ANKA. In

addition, MVs isolated from infected mice transferred the receptor to *ABCA-1-/-* mice causing infection by the parasite and development of CM[87, 88].

1.7.1.3 Exchange of genetic information by transfer of mRNA, miRNA and proteins

Epigenetic or nuclear reprogramming is defined as exchange of membrane and cytoplasmic components between different cell types when they are co-cultured which results in certain functional and molecular changes (reprogramming of adult cells into pluripotent cells)[89]. Some groups reported induction of epigenetic changes in target cells when co-cultured with cell extracts[90, 91].

Experimental evidence demonstrated that terminally differentiated cells are reprogrammed when they were subjected to extracts from embryonic stem cells (ESC), pluripotent cancerous cells or differentiated somatic cells like, cardiomyocytes[92]. In a model of murine ESC-derived microvesicles, MVs contributed to epigenetic reprogramming of target cells. In the presence of ESC-derived MVs, survival and expansion of murine haemopoietic stem and progenitor cell (HSPC) is significantly increased and upregulated expression of markers for early pluripotent and early haemopoietic stem cells is detected[92, 93]. It was also confirmed that these ESC-derived MVs were highly enriched in mRNA for various pluripotent transcription factors compared to parent cells, and this mRNA could be delivered to target cells and translated into corresponding proteins[91, 92].

1.7.1.4 MVs transfer prions and mitochondria

A 'Trojan horse' mechanism has been described to explain the direct transfer of HIV into cells by MVs[94]. Moreover, the ability of MVs to transfer prions has been suggested in platelet-derived MVs with spread of infection as an outcome[95]. Interestingly, co-culture of mitochondrial DNA- (mtDNA-) mutated cells, named A549 p° cells (impaired aerobic respiration) with human skin fibroblast, demonstrated that mutated cells were capable of aerobic respiration and this ability was acquired from fibroblast cells. This phenomenon implied a possible involvement of MVs[96].

1.7.2 MVs contribute to body self-defence

Eukaryotic cells shed MVs in response to sheer stress, tissue regeneration and inflammatory stimuli. The latter MVs carry molecules such as Fas and caspase-1 and transfer them to neighbouring cells and avoid apoptosis[97]. Shedding MVs aids cells to escape from complement-mediated lysis and subsequently removing inserted membrane attack complex (MAC). Previous works suggested that PS-induced phagocytosis of cells is inhibited by release of PS+MVs from cells[97, 98].

1.7.3 Selective secretion of proteins and RNAs through MVs shedding

MVs may transfer proteins such as cytokines, chemokines and growth factors to target cells, modulating the constitutive properties of the respective cells. Plasma membrane in eukaryotic cells plays a vital role in cell survival by separating the cytosol from the extracellular environment. A complex

endomembrane system in all living cells allows the formation of organelles[99]. These organelles contribute to the storage of materials, distribution of nutrients, exportation of substances to extracellular environment, and degradation of macromolecules[100].

Living cells require intracellular export and secretion mechanisms, as well as endo- and exocytosis in order to transport and exchange materials between intracellular organelles and the extracellular matrix[100, 101].These processes are mediated by coated vesicles (e.g. MVs), which deliver their contents upon fusion with a target membrane[102-104]. It was shown that glioblastoma cells shed MVs which carry proteins, mRNAs and miRNA. These MVs transfer their components to healthy target cells and change their translational profile. This phenomenon affirms the importance and potential physiological role of microvesicles in brain tumourigenesis.These strictly regulated mechanisms are involved in secretion, protein biogenesis, sorting, and modification[105].

1.7.4. Conventional protein secretion pathways

The endoplasmic reticulum (ER) is considered as a translocation machinery in eukaryotic cells. There are three distinct classes of proteins involved in this translocation machinery: lysosomal proteins, secretory proteins both translocated across the ER and finally, integral membrane proteins which are integrated into the ER membrane[106]. Protein secretion is initiated with insertion of a preprotein into the lumen of the ER by binding of signal

recognition particle (SRP) to an N-terminal hydrophobic, signal sequence of a nascent polypeptide chain, synthesized in the cytosol. The preprotein is completely synthesized on cytosolic ribosomes before being translocated to allow membrane-associated ribosomes to direct the nascent polypeptide chain into the ER concomitant with polypeptide elongation[107].

In both cases, preproteins are targeted to the ER membrane through specific interactions with cytosolic and/or ER membrane factors. The preprotein is then transferred to a multiprotein translocation machine in the ER membrane that includes a pore through which the preprotein passes into the ER lumen. The energy required to drive protein translocation may derive either from the coupling of translation to translocation (during cotranslational translocation) or from ER lumenal molecular chaperones that may harness the preprotein regulating the translocation machinery (during posttranslational translocation) [108]. Once localised to the lumen, the polypeptide is correctly folded by association with ER resident chaperones (a quality control measure), before being packaged into membrane-coated transport vesicles, which are approximately 50 nm in diameter. The vesicles fuse with the Golgi apparatus to deliver their protein cargo to the plasma membrane. This transportation is along a microtubular network by post-Golgi transport carriers, which fuse with the membrane and eventually release their content into the extracellular space[109].

Inhibitors such as fungal metabolite brefeldin A (BFA) inhibits ADP-ribosylation factor 1 (ARF-1) recycling by disrupting the interaction of ARF with its guanine exchange factor (GEF). This impairs formation of COPI vesicles (transport intermediates involved in recycling of proteins from Golgi to ER) and results in fusion of the cis- and medial-Golgi with ER[110].

1.7.5 Unconventional pathway

In eukaryotic cells, protein translocation into the endoplasmic reticulum (ER) is the first step in the biogenesis of most extracellular and many soluble organelle proteins (classical secretory pathway)[108]. Numerous numbers of secretory molecules despite lacking the classic N-terminal, hydrophobic, signal sequence, can still be exported by cells to the extracellular matrix. These proteins do not associate with the classical secretory pathway[108]. For example, secretion of galectin-1 was still observed, despite the absence of a functional ER/Golgi membrane transport system[111]. This new process is termed as unconventional protein export, to distinguish secretory mechanisms that are independent of the classical ER/Golgi transport pathway[112]. Further characterisation of these molecules revealed common properties for them in spite of being structurally and functionally unrelated. For example, treatment of cells with the fungal metabolite brefeldin A (BFA), an inhibitor of the ER/Golgi transport system, does not inhibit their transportation into the extracellular space[113].

In summary, unconventional secretion is a strictly regulated mechanism that is not based on unspecific protein release. It can be grouped into four main mechanisms: the first mechanism involves multivesicular bodies (MVBs), which carry exosomes packaged with cargo molecules that are released into the extracellular matrix, when MVBs fuse with the plasma membrane[114]. Galectin-3 is an example of a protein unconventionally secreted via this route[63].

The second mechanism is shedding of microvesicles into the extracellular space[4, 6, 115]. A variety of proteins are translocated into the extracellular space via this process. Among these, the galectin family and other unconventionally secreted molecules not previously shown are mediated through MV release. The third mechanism involves recruitment of endocytic membrane vesicles such as secretory lysosomes to the cell membrane, and this is exemplified in the export of IL-1β[111].

Lysosomal contents are usually translocated to the exterior of cells under favourable conditions, when specialised intracellular structures such as lysosomes of cytotoxic T lymphocytes or melanosomes of melanocytes fuse with the plasma membrane[116]. A forth mechanism characterised by direct translocation of leaderless proteins using resident-protein mediated channels, such as adenosine triphosphate-binding cassette (ABC) transporters has also been reported[117]. An example of this process is the

non-classical export of the hydrophilic acylated surface protein B (HASPB) of *Leishmania*[118].

1.8 MVs in diseases

MVs are detectable in the blood under normal physiological conditions and involved in modulation of immune response, inflammation, regulation of apoptosis, and coagulation[1]. MVs are increased in cases of cancer, inflammation, diabetes, pre-eclampsia, hypertension, prothrombotic states such as thrombotic thrombocytopenic purpura, the antiphospholipid antibody syndrome, Systemic lupus erythematosus (SLE), multiple sclerosis, cerebral malaria and many diseases associated with inflammation[18, 69, 119, 120]. MVs derived from platelets are elevated in patients with type 2 diabetes mellitus[121]. In addition, MVs have been reported to play a vital role in the development of cardiovascular diseases, rheumatoid diseases, as well as in sickle cell anaemia and even in pregnancy[122-124]. Similarly, rapidly growing cell lines tend to secrete more microvesicles than those with slower proliferation rates[120].

The presence of MVs provides important information to measure the protective effect of therapeutic intervention in a non-invasive manner as well as acting as an indicator of severity of disease states[18]. The quantity and composition of released MVs may vary in different pathologic conditions. For instance, monocyte, platelet and endothelial-cells derived MVs are found in septic patients[24].

Platelet-derived MVs are increasingly present in patients with peripheral artery disease, myocardial infarction, atherosclerosis, diabetic retinopathy, paroxysmal nocturnal haemoglubinuria and those undergoing ischemic events[24]. Moreover, recent studies suggest that cancer metastasis is enhanced upon contact with cancer cell-derived MVs[70]. Based on the mechanisms of MV release, it is possible that cytosolic and even nuclear proteins from parental cells are present within the MVs[24].

MVs can also bind to coagulation factors and promote coagulation, and induce production of prostaglandins[26, 125]. Furthermore, MVs derived from the human prostate carcinoma cell line, DU-145, stimulated endothelial cell migration[120]. MVs are also known to be elevated in acute coronary syndromes, end-stage renal disease and all conditions associated with endothelial injury or dysfunction[126]. Elevated levels of circulating platelet, monocyte, or endothelial-derived MVs are associated with most of the cardiovascular risk factors and appear indicative of poor clinical outcome[126, 127]. In addition to be a valuable marker of vascular cell damage, MVs are directly involved in atherothrombosis processes by implying direct effects on vascular or blood cells[126, 128].

Moreover, Platelets derived MVs act as a mobile transporters of tissue factor and play a significant role in maintaining haemostasis when platelets are functionally impaired in thrombocytopenia[48]. MVs also play a significant role in viral infection. Many virally infected cells secrete microvesicles that differ in

content from their virion equivalents but may contain various viral proteins and RNAs[94, 129, 130].

Drug resistance is a major cause of cancer treatment failure, with multidrug resistance (MDR) being the most serious, whereby cancer cells display cross-resistance to structurally and functionally unrelated drugs[131]. MVs have been shown to aid in the dissemination of the multi drug resistance phenotype[132]. This thesis will concentrate on the emerging role of MVs in viral infection and cancer drug resistance.

1.8.1 MVs and viral infection

Multiple cellular events such as cell death, hypoxia, stress, expression of oncogenes, differentiation, and viral infections induce microvesiculation[5]. Many reports have demonstrated involvement of viral infections in MV release. The similarities of biogenesis of enveloped viruses such as retroviruses and MVs resulted in the postulation of the Trojan exosomes hypothesis of HIV assembly and cell-cell spread. This hypothesis described how retrovirus was adapted to use host exosome machinery for the formation and transfer of virions through a non-viral route[94]. Conversely, it has been documented that exoxomes are not involved in HIV budding from plasma membrane although the endosomal sorting complex required for transport (ESCRT) of components are recruited at the sight of budding. This was confirmed when ceramide inhibition which halts exosome shedding did not affect HIV budding, in spite of a decrease in infection[64, 114, 133].

Moreover, Rhabdoviruses, Filoviruses, Arenaviruses, Herpesviruses, HBV, and HCV which belong to enveloped viruses utilize exosome machinery for their formation and spread[133]. More intensive research is required to establish inhibition of MV and virus release as novel targets to tackle infection. Exosomes have almost the same size and density as viruses which make it a challenge to study secreted MVs during viral infection[134]. Recently, development of new purification method utilizing iodixanol density gradients and immunoaffinity isolation, led to separation of pure exosomes from HIV virions[135]. HIV-derived exosomes carry co-receptors for HIV and induce virus entry into the target cells. Negative regulatory factor (Nef) protein expressed on HIV induces shedding of exosomes. The latter vesicles expressing Nef protein enhance cell death in CD4+Tcells. The significant role of exosomes machinery in evasion of immune system appears by vesicular transfer of Nef to target cells[136]. However, infected cells shed viral antigen-bearing exosomes may activate innate immune response[137]. The similarities between enveloped viruses and microvesicle biogenesis have contributed to our understanding of microvesicles biology[12].

Coxsackievirus B1 (CVB1) is a member of *Picornaviridae* family, which are among the most common human pathogens. CVB1 is a non-enveloped, single-stranded RNA virus associated with a broad spectrum of human diseases including myocarditis, meningoencephalitis, pancreatitis and paralytic myelitis. It has been reported that picornaviruses are able to depolymerise the host cytoskeleton during infection aid spread of virus and

cause disruption of actin cytoskeleton leading to release of MVs[138]. In this study, the role of MV release and spread of CVB1-induced apoptosis has been investigated.

1.8.1.1 Coxsackievirus B1 infection

Picornaviruses are small, non-enveloped, icosahedral, single stranded-RNA viruses. Coxsackieviruses, polio virus (PV), human rhinoviruses (HRV), foot-and-mouth disease viruses (FMDV) and hepatitis A virus (HAV) are members of this family[139]. The coxsackievirus was first reported in Coxsackie, New York in 1948. There are six coxsackieviruses B (CVB) serotypes, each responsible for different symptoms and diseases. CVBs cause central nervous system infections in infants and children, as well as heart muscle infections in both children and adults[139]. These viruses are the most common cause for myocarditis (inflammation of the muscular walls of the heart) and dilated cardiomyopathy. Entroviruses such as coxsackieviruses and adenoviruses have been implicated as causes of myocarditis. A significant data in murine models have shown molecular and cellular mechanisms associated with coxsackieviruses[140].

1.8.1.1.1 Mechanisms of viral entry into the cells

In order to infect, viruses confront a number of barriers to entry such as glycocalyx which blocks virus access to the cell surface. Polarized epithelial cells locate receptor molecules to the basolateral cell surface where they are not accessible to viruses, and specialized cellular junctions make the

epithelium impermeable[141]. The formation of virus-cell receptor complex results in activation of cellular signalling cascades and ligand triggered processes such as calveolar/raft endocyosis, clathrin-coat assembly, and actin cortex dissociation[142]. Viruses only infect cells with specific viral receptors. Many viruses are known to use more than one type of receptor, either in parallel or in series. For example, HIV1 binds to glycosylceramides and heparan sulfate, interactions that may facilitate the initial recruitment of virus to susceptible cells[129].

Moreover, the presence of specific glycosphingolipids in the target cell membrane can enhance CD4/coreceptor-dependent fusion[85]. Some viruses undergo rapid mutations and may switch receptors or even use alternative receptors. This poses a great risk by avian influenza to interact with glycoconjogates on human cells. Release of viral genome into cytosol is called penetration[143]. Non-enveloped viruses use pore formation mechanism to release their genome. By contrast, it involves membrane fusion for enveloped viruses[144].

Coxsakievirus B (CVB) with six serotypes and adenoviruses share the same receptor named coxsackievirus and adenovirus receptor (CAR) for cell entry. CAR is a glycoprotein with 45kDa molecular weight, a transmembrane receptor with 107 amino acid cytoplasmic domain and two extracellular immunoglobulin domains (D1and D2). CAR is member of intracellular adhesion molecules and vascular cell adhesion molecules receptors family

and functions in both attachment and infection of CVB. In adenovirus infection, CAR is only involved in attachment and integrins are associated with the entry of virus into cells[145]. In addition, rhinovirus and encephamyocarditis virus (members of Piccornaviridae family) use CAR for cell entry[146]. The extracellular region of CAR binds to another CAR molecule located on an adjacent cell as an anti-parallel homodimer[143].

CAR is expressed in many tissues and is highly conserved between mice and humans. In addition, CVBs use DAF (GPI-anchored protein) and adenoviruses uses integrins as co-receptors to infect[144]. Coxsackieviruses interact with specific receptors on the cell surface termed cell-surface molecules which belong to the immunoglobulin super family of proteins (IgSF). IgSF is comprised of two to five Ig-like domains. The transmembrane C-terminal section with short cytoplasmic region and N-terminal domain (D1) interacts with invading viruses. However, low-density lipoprotein receptors (LDL-R) are not members of IgSF family; they bind outside the canyon and are unable to initiate viral infection[147].

Moreover, Picornaviruses have canyon-like receptors on their surface to attach to relative cellular receptors. It is required for the virus to be destabilized to initiate infection and this is aided by binding into the canyon leading to the viral uncoating process. If virus uses non-IgSF molecules, binding occurs outside the canyon and viral uncoating is not caused[143]. Infection only occurs if a receptor is able to initiate the full viral life cycle.

Canyons are located on the surface of viruses and they encircle each of the twelve five-fold vertices. They protect the virus from neutralizing antibodies and immune surveillance[148].

In epithelial cells, CAR is located at the tight junctions, protein complexes that are in charge of the selective passage of ions and molecules across the epithelium and therefore are not accessible to incoming viruses from the apical side. CVBs interact with co-receptors present on the apical surface of epithelial cells, the decay accelerating factor (DAF). Virus binding leads to crosslinking of DAF molecules and activation of the tyrosine kinase c-Abl. CVBs induce lateral movement along the membrane and bind to filopodia and surf on the outside of it to reach CAR receptor meanwhile c-Abl is activated upon interaction of virus-DAF receptor. The actin cytoskeleton and c-Abl activity are required for CVB entry and infection[148, 149].

Once activated, c-Abl activates the Rho family small GTPase Rac, which in turn induces a rearrangement of the actin cytoskeleton and promote transfer of bound viruses from the apical surface to the tight-junction region of the cell. This makes it possible for the virus to associate with CAR and for CAR to induce a conformational change in the virus particle, uncoating, and entry[139]. Upon entry, virus releases its RNA through caveolar endocytosis which is activated by phosphorylation of Tyr14 in caveolin-1 by Fyn, a member of the Src family of nonreceptor tyrosine kinases. ER is the site of this release. CVB replicates in cytosol and do not need low pH to

penetrate[141]. The level of CAR expression is an indicator of susceptibility to viral infection in young children. Upon virus entry immune system response is to eliminate the virus otherwise chronic myocarditis can evolve[143]. Cytokines are important mediators of the innate immune system in response to infection. Interferons are compromised of two types: type I including interferon α and β, type II consists of interferon- γ. Both types of inteferons inhibit viral replication *in vitro*. In addition, upon virus entry, single strand RNA is released and used as a template for viral genome to replicate[150].

Earlier reports have described that CVB1-induced apoptosis is a viral mechanism to aid maximum virus dissemination[144, 151]. While the exact process(s) involved remain unclear, some studies have suggested that CVB1 induction of apoptosis in neighbouring cells is not exclusively caused by the lytic escape of enteroviruses[152, 153]. In addition, earlier studies have postulated a direct cell-to-cell spread of the poliovirus in the central nervous system[154]. Moreover, a recent study has also described a nonlytic viral mechanism of cell-to-cell transmission that involves CVB3 induction of cellular protrusions[130].

1.8.1.1.2 Mechanisms of virus internalization and release

Epithelial cells have both apical and basolateral surfaces. CVBs interact with the GPI-anchored protein decay-accelerating factor (DAF) on the apical cell surface which in turn activates Abl kinase, triggering Rac-dependent actin rearrangements that permit virus movement to the tight junction. Tight

junctions located in basolateral surface, are involved in tightly regulated passage of ions and viruses, and contain transmembrane component CAR which promotes conformational changes in the virus capsid that are essential for virus entry and release of viral RNA[142].

Furthermore, interaction with DAF also activates Fyn kinase, an event that is required for the phosphorylation of caveolin and transport of virus into the cell within caveolar vesicles and caveolar endocytosis[155]. Clatherin-mediated, rapid endocytic pathway is a mechanism for internalizing most of viruses. It transports incoming viruses together with their receptors into early and late endosomes[156]. Acidic pH inside the endosomes results in virus changes and penetration. Transmission of viruses that are pH-independent is through fusion of proteins expressed on the surface of infected cell with neighbouring uninfected cell expressing appropriate receptors leading to formation of heterokaryons. This facilitates dissemination of the virus without the formation of mature virus particles. However, transfer of viral genomes from cell to cell occurs upon formation of virus particles which are released through formation of multi-nucleate enlarged cells called syncytia[157, 158]. Furthermore, the virus life cycle consists of assembly, dissemination and entry into new cells where cell free viruses exposed to the humoral immune system remains a minimum and they can be protected[159].

Enveloped viruses are released through budding from infected cells. Non-enveloped viruses use cell lysis for release. It has been documented that

some non-enveloped viruses after budding through membrane compartments, lose their membrane or some gain access to exocytic organelles and are released[150]. Exosomes have a crucial role in dissemination of viruses and spread of infection. It has been shown that HIV manipulates host exosomes to propagate infection[160]. Epstein-Barr virus (EBV) positive B cells release exosomes which contain viral microRNAs. These exosomes transferred these miRNAs to monocytes. In addition, non-infected B cells were positive for EBV miRNAs[161].

Exosomes have a potential to transfer viral genetic factors to non-infected cells[162]. A previous study has shown that in infectious prion diseases such as Kuru, exosomes carried prion protein scrapie (PrP^{sc}) and deliver it into non-infected cells which shows a possible contribution of exosomes in prion propagation[163]. Finally, exosomes play a significant role in the biology of infectious diseases and act as secretory organelles with complex roles in cell-to-cell communication and immune surveillance[164].

1.8.1.1.3 Pathophysiology

As mentioned before, members of the CVB group are considered the main cause of virus-induced myocardial disease, infecting a wide range of cells in the immune system. CVBs are causal agents of the common cold, pleurodynia, aseptic meningitis upper respiratory infection, myocarditis and pericarditis. Coxsackie B viruses have also been known to cause: encephalitis, flaccid motor paralysis, exanthema, rash, pneumonia and

generalized disease of the newborn. Viral myocarditis may lead to heart failure and permanent heart damage. Some patients might be paralysed for the rest of their life[159].

1.8.1.1.4 Symptoms

The symptoms are ranging from mild to severe, but mostly mild symptoms are common. The incubation period for CVBs is 2-10 days. Fever, malaise, fatigue, chest pain are relative symptoms. Cardiac symptoms and heart damage occur two weeks after infection[158].

1.8.1.1.5 Diagnosis

Examination of patients and checking for the characteristic symptoms, laboratory tests on faeces and throat sample to isolate CVB are the main diagnostic tests[139].

1.8.1.1.6 Prevention

CVB transmitted through faecal-oral mode. It is also transmitted by contacts with infected mucosal secretions, contaminated surfaces. It is impossible to prevent CVB and general sanitary measures such as frequent hand washing and avoidance of contaminated water must be taken[139].

1.8.1.1.7 Treatment

Certain medications are prescribed in order to ease the symptoms[156].

1.8.2 MVs in cancer

In the 1970s, the presence of MVs in blood samples from cancer patients was reported[1]. The reason underlying elevated levels of MVs in cancer patients is unknown but in a mouse model, increased release of Tissue factor (TF)-bearing MVs was associated with the loss of tumour suppressor gene p53[48]. Since cancer patients have elevated levels of MVs and procoagulant platelet-derived microvesicles in the blood, the procoagulant state of cancer patients has partly been attributed to these MVs[165]. Cancer patients with venous thromboembolism carry higher levels of TF-bearing MVs, in contrast to those without thrombosis[48, 165]. Thus, this phenomenon indicates that MVs are involved in cancer progression and biology[18].

Moreover, heterogeneous MVs released by cancer cells are capable of merging with recipient cells to transfer their cargo[1]. Tumour derived-MVs contain growth factors, receptors, proteases, adhesion molecules, signalling molecules, as well as DNA, mRNA, and microRNA (miRNAs) sequences[11]. MVs contain intracellular proteins, second messengers, genetic material which specifically sorted into microvesicles. As a consequence of sorting, the functional properties and biological role of microvesicles may differ from their parental cells. These contents can be transferred to non-transformed stromal cells, endothelial cells, and impact tumour invasion, angiogenesis, metastasis, and drug resistance[18].

1.8.2.1 Role of MVs in cancer progression

MVs might be involved in the interactions among host cells and tumour cells as well as bone marrow-derived cells. MVs derived from tumour cells and hematopoietic cells are thought to mediate the formation of metastatic niches[70]. MVs are secreted from the primary tumour which then attracts hematopoietic cells to the primary tumour. Tumour-derived microvesicles secreted by tumor cells induce endothelial cells to release microvesicles that contain vascular endothelial growth factor (VEGF) and sphingomyelin in order to promote angiogenesis. The adverse tumor microenvironment somehow triggers tumor cells to release more microvesicles, which in turn facilitates angiogenesis by bringing nutrients and oxygen to the rescue of cancer cells. In the primary tumour, the microenvironment could then be developed by MVs secreted by platelets carrying platelet-derived growth factor BB (PDGF-BB), thus promoting a tendency to malignancy by the tumour cells[74]. It has been shown by Castellana et al.(2009) that microvesicles released by PC3M cells, an invasive prostate cancer cell line, triggered ERK phosphorylation, MMP9 upregulation, increased motility and resistance to apoptosis in fibroblasts in the surrounding microenvironment. In turn, the activated fibroblasts shed microvesicles to facilitate the migration and invasion of the prostate cancer line[119]. Finally, MVs from hematopoietc stem cells could then attract tumour cells to locations in the bone or other metastatic sites thus initiating the development of secondary metastatic sites[76].

1.8.2.2 The involvement of MVs in escape from apoptosis

MV release removes intracellular stress and act as a protective mechanism for cell survival[97]. Cells maintain internal equilibrium by adjusting their physiological processes by apoptosis. Apoptosis or programmed cell death is a tightly regulated process which has a crucial role in development and tissue homeostasis[166]. Caspase-3 is one of the main executioner enzymes of apoptosis. MVs bearing caspase-3 were isolated from conditional medium of viable cells. However, caspase-3 was undetectable in cells from which these MVs are emanated[123]. This was further confirmed in another study when in caspase-3 deficient MCF-7, inhibition of MV release was restored by transfecting cells with caspase-3. Various studies have documented that cancer cells prevent accumulation of intracellular caspase-3 by releasing caspase-3 bearing MVs[167]. This hypothesis was supported by evidence that inhibition of MV release results in accumulation of caspase-3 inside cells leading to apoptosis. Therefore, release of caspase-3-containing MVs contributes to cell survival[168].

Many investigations have demonstrated an association between MV release and multi-drug resistance. To further illustrate the correlation between MVs shedding and cancer cells survival, a study by Shedden showed that chemo-insensitive cancer cell lines express more membrane shedding-related genes compared with chemo-sensitive cells. In addition, MVs released from chemo-insensitive cancer cells contained higher levels of the chemotherapeutic agent doxorubicin[169]. Safeaei and colleagues (2005) have also demonstrated

that exosomes from cisplatin-insensitive cancer cells contained 2-6 folds more of the chemotherapeutic drug cisplatin than MVs released from the cisplatin-sensitive cells[170].

1.8.2.3 MVs and escape from immune system

Complement activation induces the release of MVs[171]. Sims and co-workers (1988) demonstrated that apoptosis was abrogated in human platelets which were co-cultured with a sublytic concentration of the membrane attack complex, C5b-9(MAC) due to release of C5b-9-bearing MVs. This phenomenon referred to as 'complement resistance' is a way by which MV release offers protection to most cells in response to exogenous stimuli. The same mechanism aids cancer cells to escape from complement lysis[2]. Cancer cells also shed MVs bearing the complement membrane cofactor protein, CD46, which inactivates C4b and C3b and therefore results in reduction of inflammation in micro-tumours[168]. To induce T-cell apoptosis and impair adaptive immune system, cancer cells enhance their survival by expression of Fas ligand (FasL, CD95L) a ligand of the death receptor, Fas (CD95)[82]. Moreover, cancer cell-derived MVs fuse with plasma membrane of monocytes and impair their differentiation to dendritic cells[60]. It has been demonstrated that in tumours developed in patients who suffer from Epstein-Barr virus infection, released MVs express Latent membrane protein-1(LMP-1), an immune suppressor transmembrane protein which restrain T-cell proliferation and therefore impair immune system function[161]. Furthermore, differentiation of monocytes to dendritic cells, an antigen presenting cells, is

inhibited by tumour derived-MVs. Cancer cells may escape from the immune system by mimicking the host environment[172].

1.8.2.4 MVs contribute to metastasis and angiogenesis

MVs contain proteases such as matrix metalloproteinase (MMP)-2 and MMP-9, which degrade collagens and facilitate tumour growth. Inhibition of MMP-2 and MMP-9 presented in MVs abolishes the ability of these MVs to support tumour invasiveness[173]. Tumour derived-MVs also carry urokinase-type plasminogen activator (uPA) which catalyses plasminogen into plasmin reaction. Plasmin debases fibrin and as a result extra cellular matrix is degraded which contributes to tumour growth[174]. Cancer cells are also coated with fibrin and escape from immune detection and attack, whilst the fibrin matrix supports outgrowth of new blood vessels simultaneously[69]. MVs in cancer patients contain TF, which contributes to thrombosis. The latter MVs are trapped by activated platelets and transfer and compile their procoagulant TF at the site of damage[48].

Furthermore, these MVs may merge with platelets leading to coagulation and emission of growth factors[123]. Cancer cell-derived MVs carry mRNA encoding growth factors such as vascular endothelial growth factor (VEGF) and these vesicles fuse with monocytes and transfer their nucleic acids and induce production of growth factors[175]. Cancer cell-derived MVs alter functions of T cells and show immunosuppressive activities[18]. The fact that MVs suppress immune system, might contribute to tumour cell metastasis and spread. This

spread might be haematological through TF-bearing MVs originated from activated platelets which express and carry P-selectin glycoprotein (PSGL)[176]. Cancer cells surrounded by these MVs are protected from immune system scrutiny. Moreover, procoagulant properties of TF-bearing MVs lead to intravascular formation of fibrin aid adherence of cancer cells to vessel wall[168].

By contrast, cancer cell-derived MVs transfer tumour antigens to antigen-presenting cells and facilitate immune attack. Antigen-presenting cells also release MVs and the latter vesicles suppress growth of murine tumours[177]. Anti-cancer therapy targets release of MVs, i.e. by counteracting the beneficial effects of release of MVs on tumour growth. Rock-I and II are serine-threonine kinases and both affect cell morphology, migration, cell adherence and release of MVs[120] .

The therapeutic role of dendritic cells derived-MVs in metastatic melanoma, advanced non-small cell lung cancer and colorectal cancer has been examined and may be promising for cancer therapy[69]. Inhibition of MV release could be a potential target in anti-cancer therapy because MV release is associated with many processes related to tumour growth[78]. Another study illustrated that heat treated B-lymphoblastoid cells (42°C) release exosomes of differing protein composition from control MVs containing high quantities of heat shock protein[114]. The efficacy of anticancer drugs can be examined through proteomic studies of cancer cell-derived MVs

which might indicate the effects of chemotherapy and could be considered as an early biomarker for assessment of drugs. Proteomics can also be used in bladder cancer where MVs were shown to have eight proteins at elevated levels compared to control[63]. Cancer specific-mRNA or microRNA could be other markers to be considered for early detection of cancer[178]. MVs quantification before administration of chemotherapeutic agents could be considered as an indicator of survival rate in cancer patients.

To examine the efficacy of anti-cancer drugs, the measurement of the protein composition of MVs could be an early biomarker to assess the effectiveness of anti-cancer therapy[1]. Circulating MVs expose tumour-specific markers, which could be useful for early detection of cancer. Moreover, detection of cancer-specific mRNA and microRNA could be used in patients with a high risk for cancer[67].

1.8.2.5 Drug resistance

Drug resistance in solid tumours has been a hitherto unsolved pharmacological problem in cancer chemotherapy. There are two types of drug resistance: intrinsic (inherent) resistance, where resistance to chemotherapy already exists before starting a drug treatment program, and acquired resistance, which develops during treatment[131]. In order to tackle this problem new drugs that can specifically target cancer cells were developed. However, these drugs have not decreased the occurrence of drug resistance so far[179]. Inefficient treatment of patients would be avoided by

early diagnosis of drug resistance. In the 1950s, cytostatic drugs started to be tested to predict tumour reaction. Better techniques were developed over the last few decades. Response assessment in solid tumours is based on imaging the size of the tumour. However, early detection of drug resistance may be delayed while waiting for tumour shrinkage[180]. In order to diagnose intrinsic drug resistance, fresh cancer cell culture assays are currently available, but these methods carry a high cost. Positron-emission tomography (PET) is a test used to detect drug resistance but it cannot distinguish between inherent or acquired resistance[181].

An alternative classification for drug resistance is either pharmacokinetic (marked intratumoural differences in drug exposure) or pharmacodynamic (failure to elicit cytotoxcity)[131]. Multiple pathways are affected and act synergistically to create resistance to anti-cancer drugs. Chemotherapy is one of the major therapeutic avenues in oncology especially after dissemination of cancer which is difficult to treat with radiation[182].

In addition, chemotherapy is used as an adjuvant therapy and for palliation. Cancer cells, in response to chemotherapy, display various pathways, which interact synergistically to confound the cytotoxcity of chemotherapeutic agents[183]. Anti-cancer drugs are designed to target cell proliferation through inhibition of specific steps of the DNA replication process i.e. alteration of nucleoside biosynthesis, interaction with DNA and prevention of cell mitosis[184]. Chemotherapy results in changes in the biology of cancer cells,

which consequently leads to chemoresistance. The transcription factor p53 is an essential regulator of cell stress response, which is activated by a broad range of stimuli such as DNA damage, hypoxia, loss of cell-to-cell contact and inappropriate oncogene activation[185]. An endogenous inhibitor *Hdm2* promotes p53 degradation because p53 is a short-lived protein. Any cellular stress results in p53 stabilization leading to p53 posttranslational modification and its interaction with DNA and co-operating factors[186].

1.8.2.5.1 Association of p53 in cancer drug resistance

The p53 gene is a tumour suppressor gene. The p53 gene has been mapped to chromosome 17[187]. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot pass through to the next stage of cell division. Consequently, a chain of events is initiated that reduces the effects of damage by up-regulation of numerous genes such as *p21* which functions as a regulator of cell cycle progression at G1 and suspension of the cell cycle. Cell cycle suspension allows repair of DNA and cellular damage, thereby promoting cell viability and survival[185, 188]. If DNA damage is irreparable, p53 induces the expression of apoptosis inducers (e.g. Fas and Bax) in order to halt replication of damaged DNA in daughter cells. This p53 function duality demonstrates its critical role in cell biology by acting as a tumour suppressor gene[186]. This crucial role is reflected by observations of approximately 53% of p53 mutations in cancers. This high degree of p53 mutations compromises

the ability of p53 to regulate the cell cycle and promote apoptosis in response to cellular damage. Consequently, for p53, a striking role in dictating the effectiveness of genotoxic chemotherapeutic agents in cancer treatment is expected[185]. It has been shown for a number of anti-cancer drugs such as 5-fluorouracil (5-FU) in colorectal cancer that loss of p53 function, prevents the initiation of apoptosis following chemotherapeutic insult, thereby conferring resistance[131].

As a result, gene therapy is being investigated to increase chemotherapeutic efficacy. However, many studies have failed to confirm the link between mutated p53 and anti-cancer drug resistance[189]. There are a number of endogenous DNA repair mechanisms in cancer cells such as base excision, nucleotide excision, mismatch or direct repair of damage which are employed due to the exposure to chemicals or radiation. Depending on the nature of drug-DNA compound, a specific pathway is chosen by cancer cells to repair damage caused by chemotherapeutic agents[185].

Moreover, different tissues vary in the way they use their repair pathways such that it is even more difficult to predict chemotherapeutic drug effectuality[131]. Development of drug resistance is a result of several factors including increased drug efflux and decreased drug influx; drug inactivation; alterations in drug target; processing of drug-induced damage; and evasion of apoptosis[183]. The tumour suppressor protein p53 plays a critical role in the regulation of cell cycle arrest and cell death. The gene encoding p53, *TP53*,

is the most frequently mutated gene in human cancers, with ~50% of all tumours estimated to carry a mutation[189]. ATM (ataxia–telangiectasia mutated), ATR (ATM and Rad- 3 related), and DNA-PK (DNA-dependent protein kinase) are relative kinases which are activated by DNA damage and lead to activation of p53[190]. Moreover, p53 trigger elimination of the damaged cells by promoting apoptosis through the upregulation of pro-apoptotic genes such as Bax, NOXA, TRAIL-R2 (DR5), and Fas (CD95/Apo-1)[191].

Therefore, p53 maintains genomic integrity and prevents damaged DNA being passed on to daughter cells. Numerous studies indicated that dysfunctional p53 attributes to drug resistance due to inability to undergo apoptosis in cells treated with 5-FU. Apoptosis is the main target of chemotherapeutic drugs. Extra- and intra-cellular signals are amplified by second messengers and activate caspases[131].

1.8.2.5.2 Role of P-glycoprotein in cancer drug resistance

P-glycoprotein (P-gp) and multidrug resistance protein (MRP) are members of ABC transporter proteins family and are involved in drug efflux[189]. The first glycoprotein discovered that was responsible for conferring drug resistance to cultured cells was the permeability glycoprotein (P-gp). P-gp is an energy-dependent efflux transporter which pumps drug molecules out of cells[192]. The multi-drug-resistance-associated protein (MRP1) is involved in the resistance of lung cancer cells[193]. P-gp is found in the epithelial cells of the intestine (enterocytes) along the apical (luminal) side of the cell. When a drug is taken

orally, drug molecules have to pass through the enterocyte to pick up the molecules and carry them back to the luminal side of the cell, where they are dumped back into the lumen of the intestine. This action prevents drug molecules from reaching the systemic circulation, effectively limiting bioavailability[190]. Because P-gp is found throughout the intestinal tract, it affects the absorption of all susceptible oral drugs. P-gp also is present in the liver and kidney, where it acts to increase the excretion of drugs by transporting the molecules into the bile and urine, respectively[194]. Over expression of these proteins directly correlate to drug resistance. Multi drug resistance has been documented to be linked to over expression of P-gp. Administration of P-gp inhibitors such as cyclosporine and vaslapodar has not been successful due to their cytotoxcity targeting other transporter proteins[182].

There are other members of the ABC superfamily of transporters which are associated with conferring resistance to chemotherapy, for example the multi-drug-resistance-associated protein (MRP1) which is involved in the resistance of lung cancer cell[180].The breast cancer resistance protein (BCRP) or mitoxantrone resistance protein is isolated from cell lines resistance to mitoxantrone . Moreover, BCRP is an ABC half-transporter and translocates unmetabolised drugs. It confers resistance to a subset of drugs distinct to drugs transported by P-gp and MRP[195]. All mentioned transporters protect cancer cells from anti-cancer drugs and have a crucial role in drug resistance. P-gp = ABC $_{B1}$, MRP1= ABC $_{c1}$, and BCRP= ABC$_{G2}$ are assigned

systematic nomenclature for these three transporters. They are expressed in various normal tissues specifically in the gastro-intestinal tract, liver and blood-brain barrier with secretory/excretory function[87, 195].

For the first time, Levchenko and colleagues described an acquired non-genetic mechanism involved in multi drug resistance. They showed that co-culturing P-gp positive cells with P-gp negative cells results in direct cell-cell transfer of P-gp and expression of P-gp in recipient P-gp negative cells[196]. An elegant study by Bebawy M et al in 2009, showed the involvement of MVs in transferring P-gp from chemo-resistance cells into chemo-sensitive leukemic cells and conferring drug resistance in target leukemic cells[132].

1.8.2.6 Apoptosis

Apoptosis (or programmed cell death) occurs via extrinsic or intrinsic pathways depending on the initial signal[53]. The death receptors belonging to the tumour necrosis factor (TNF) family are involved in the extrinsic pathway leading to a cascade of events that result in activation of caspases-3 and 8. These proteases dismantle the cell and create morphological changes such as DNA condensation and degradation[54, 197].

DNA damage or numerous forms of cellular stress initiate activation of the intrinsic mitochondrial pathway leading to activation of caspases. Mainly the release of cytochrome c, due to mitochondrial depolarisation, is associated

with the intrinsic pathway[53]. The major route for chemotherapy-induced apoptosis is the intrinsic pathway. Any disturbance of this may lead to significant alterations in the response to chemotherapeutic agents[131].

Bcl-2 proteins are regulators of intrinsic pathway. Activation of proximal caspases results in activation of effector caspases such as caspase-3 and 7. These active caspases create morphological and biochemical changes including nuclear fragmentation and cell shrinkage and finally form apoptotic bodies. Some members of Bcl-2 family including Bax, Bak, and Bad promote apoptosis and result in release of mitochondrial cytochrome c[131]. Apoptosis protease activating factor (APAF-1) in junction with cytochrome c and caspase 9 form apoptosome. Formation of this complex induces cleavage of effector caspases and apoptosis[198].

However, other members such as Bcl-X_L, Mcl-1, and Bcl-2 act as anti-apoptotic proteins and inhibit release of cytochrome c and apoptosis .The balance of pro- and anti-apoptotic proteins such as the Bcl-2 family governs mitochondrial membrane integrity. For instance, over-expression of Bcl-2/Bcl-x_L proteins is associated with chemotherapy resistance, while down-regulation, with RNA interference, increases the drug response. Clinical data gathered from acute myeloid leukaemia, advanced breast cancer and non-Hodgkins lymphoma patients show this correlation[179].

On the other hand, over-expression of Bcl-2/Bcl-x_L is also related to slow proliferation[100]. Overall, disruption in the balance between pro-/anti-apoptotic factors will affect cell survival during chemotherapy. TNF-receptor family is the main regulator of extrinsic pathway. Fas (CD95/APO-1) is a member of TNF-family[81]. The role of Fas (CD95/APO1) has been studied extensively and it has been shown that formation of Fas-Fas ligand complex lead to recruitment of caspase-8 via the adapter molecule FADD (Fas-associated death domain) and formation of a death inducing signalling complex (DISC). Consequently, caspase-8 activates caspase cascade resulting in apoptosis[199].

However, in some cells BID a member of Bcl-2 family is cleaved by caspase-8 and translocates to the mitochondrial membrane leading to release of cytochrome c[131]. Several studies suggested that Fas is crucial mediator of response to chemotherapy in colorectal tumours treated by 5-FU. Moreover, some reports have shown that up-regulation of FasL and down-regulation of Fas is indicator of cancer progression when tumour FasL positive attacked Fas expressing immune cells[82]. The protein tyrosine kinases (PTKs) are a large and diverse multigene family which are important regulator of cell to cell signalling in physiological processes such as growth, differentiation, adhesion, motility, and death. PTKs play a significant role in drug resistance through regulation of anti-apoptotic signal transduction pathways[189].

The most characterized members are epidermal growth factor receptor (EGFR) family, which comprise EGFR (ErbB1, Her1), Her2 (ErbB2, Neu), Her3 (ErbB3), and Her4 (ErbB4). Over expression and oncogenic mutations of many PTKs have been described in human cancers[189].

These tyrosine kinases activate antiapoptotic signals including the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB) pathway and the STAT (signal transducers and activators of transcription) pathway. Several reports described correlation between cancer and overexpression of EGFR where receptor overexpression is associated with advanced disease and poor prognosis. *In vitro* data demonstrated that high expression of EGFR and Her2 contribute to increase cancer drug resistance and inhibition of these receptors has been examined in clinical trials[200].

DNA is a frequent target for chemotherapeutic agents and its methylation is associated with transcriptional inhibition. Moreover, tumour suppressor genes are also targets of methylation. The caspase-8 promoter is another observed target for methylation, which ultimately decreases apoptosis. Therefore reversing this process is an important therapeutic approach[201].

Drug resistance mechanisms compromise a number of pharmacokinetic properties of drugs such as reduction of exposure of cellular targets to active drug species so that the tumour demonstrates a pharmacokinetic resistance to chemotherapeutic agents[131]. A significant proportion of cancer cells are

located at a short distance from the nearest blood vessel. This factor has a crucial role in generating a local micro-environment and provides an acidic pH and hypoxia. In addition, the aberrant structure affects the intratumour pharmacokinetics of drugs leading to poor drug distribution and failure to expose the most distal regions to chemotherapy[202]. In many tumours, the lack of functional vessels prevents drugs from complete distribution. Following administration of chemotherapeutic agents, the plasma membrane of the cells comprising the tumour represents the first barrier, which impedes drug efficacy[189].

1.8.2.7 Mitotic Inhibitors

1.8.2.7.1 Taxanes

A class of chemotherapeutic drugs derived from the European yew tree is called taxanes. Paclitaxel and docetaxel are the most important members of this group as they have antimitotic properties which inhibit microtubule polymerization and halt mitosis at the metaphase-anaphase and perturb mitotic spindle formation leading to apoptosis therefore changes in microtubule polymer mass and expression of various microtubules are associated with development of antimicrotubule resistance[181].

Cell cytoskeleton is compromised of microtubules fibres, α- and β subunits which play significant role in cell signalling, cell division, mitosis, cell shape, transportation of vesicles and mitochondria[42]. Polymerisation of microtubules is essential for successful spindle function and chromosomes segregation in

mitosis. The mitotic spindles consist of polymerised α- and β-tubulin. Taxanes such as paclitaxel and docetoxel are bound to the β-tubulin and stabilise microtubule and inhibit depolymerisation of spindles thereby arresting the cell cycle and induce apoptosis[181].

The taxanes are widely used as a treatment of solid tumours of the breast, ovary, prostate and lung. Taxanes are used either as a single drug or in combination with other anti-cancer drugs in breast cancer. Gradually, docetaxel becomes prominent and preferred over taxol[181]. However, clinical resistance to taxanes is significant and 30-50% response to docetoxel is observed in metastatic breast cancer[181]. Reduced cellular accumulation and target alteration are the most prominent mechanisms involved in taxane resistance. Moreover, transporters ABC_{B1}, ABC_{B11} (bile salt export pump) and ABC_{C1} have been implicated in this taxane resistance[87]. Inhibitors such as verapamil and cyclosporine A are able to restore docetoxel efficacy in various cell lines *in vitro*[131]. Alterations in the activity of particular drug targets, in the case of taxane resistance mutation or alteration in expression of the various β-tubulin protein isotopes have a major contribution in chemoresistance[179].

1.8.2.8 Anti-metabolites

1.8.2.8.1 5-fluorouracil

Anti-metabolite compounds are a widely utilised class of chemotherapeutic drugs. 5-fluorouracil (5-FU) a pyrimidine analogue is processed by the cell in

the pyrimidine synthesis pathway which results in formation of inhibitor (5-fluoro-2′-deoxyuridine-5′-monophosphate, FdUMP) of thymidylate synthase (TS). This inhibition leads to depletion of thymidine, which arrests cell replication in the S phase of the cell cycle. Therefore there is an inverse correlation between 5-FU efficacy and the expression level of TS resulting in resistance to the chemotherapeutic agent which is caused by restoration of the nucleoside synthetic pathway[131].

An irreversible inhibitor of thymidylate synthase 5-FU is anti-metabolite drug degraded in liver by dihydropyrimidine dehydrogenase (DPD). Resistance to 5-FU has been described because of over expression of DPD in cancer cells. Moreover, colorectal tumours resistance to 5-FU is due to over expression of relative DPD mRNA tumour cells. 5-FU causes indirect DNA damage and cells either repair this damage or undergo apoptosis[183].

1.8.2.8.2 Methotrexate

Methotrexate (MTX) is an inhibitor of dehydrofolate reductase (DHFR), which results in accumulation of dehydrofolate and more critically, inhibition of TS and nucleotide biosynthesis[203]. It is prescribed in treatment of lymphoma, choriocarcinoma and acute lymphoblastic leukaemias (ALL)[193]. It also used in combined chemotherapy in metastatic breast, bladder and gastric cancers. Many mechanisms are responsible for MTX resistance[204]. Membrane translocation systems are in charge of the maintenance of high concentration

of intracellular folate such as prominent mechanism, an energy dependent bidirectional membrane transporter called reduced folate carrier (RFC). The RFC is ubiquitously expressed in normal and cancer cells and this expression is regulated by the cellular folate status[203].

The RFC has similar affinity for both MTX and folate cofactors for transport. The level of RFC is related to the efficacy of MTX and other anti-folates. Folate receptors (FRα or FRβ) are located at the plasma membrane and anti-folate chemotherapeutics bind to them. Inhibitors of folate pathway such as MTX inhibitor of dehydrofolate redoctase (DHFR) interact with reduced folate carrier (RFC) and enter the cells[131]. Anti-folate chemotherapeutics also enter via an endocytotic process in association with clathrin[180]. MTX enters cancer cells via RFC and the concentration gradient into the cell is maintained by conjugation with several glutamate residues and this reaction is catalysed by folypolyglutamate synthase (FPGS). Reduced level of expression or activity of FPGS is associated with MTX resistance in a variety of cell lines *in vitro*[180]. MTX resistance has also been described as the result of decreased expression of the RFC or complete inactivation of the receptor[179]. Recently it has been demonstrated that in children with chronic myeloid leukaemia (CML) who have specific RFC genotype (80AA) RFC uptakes a low level of MTX suggesting that diminished uptake by RFC develops a significant general resistance to antifolates[203].

1.9 Plasma membrane resealing

Plasma membrane (PM) consists of two layers of phospholipids and embedded proteins. Cell survival and homeostasis are the result of an intact plasma membrane[205]. Damage to the integrity of the host PM leads to activation of mechanisms such as Ca^{2+}-dependent vesicle-vesicle fusion or vesicle-PM fusion required for PM resealing[205]. Lysosomal associated membrane proteins 1 and 2 (LAMP-1 and 2) are the major integral membrane proteins of lysosomes reported to be involved in damage repair[206]. In order to survive, eukaryotic cells must maintain the integrity of the PM. Plasma membrane resealing is therefore vital to prevent premature cell death[207]. Resealing occurs within seconds of the sustained damage and it involves the exocytosis of organelles such as lysosomes and enlargosomes to the site of damage[206].

As an unrepaired PM could lead to the death of eukaryotic cells, immediate resealing of PM break up is necessary to prevent the influx of harmful substances including Ca^{2+} ions, and the loss of essential cytosolic components such as proteins and ATP[208]. In some genetic disorders the role of partial membrane repair has been implicated in symptoms of related disorders[209]. In muscular dystrophies, limb-girdle muscle dystrophy[210] is reported as a result of mutations in the gene encoding dysferlin, a 230 kD surface-bound protein essential for muscle PM repair[211] Moreover, cardiomyopathy was abrogated in mice treated with agents that elicit PM repair[208].

Two major membrane fusion mechanisms have been identified to be involved in PM resealing. The first mechanism as described before, involves the rapid exocytosis of vesicles and fusion with the PM at the site of damage[212]. The second event described recently is reserved for PM disruptions larger than 1000 μm^2, caused for example by agents such as staurosporin or insertion of the complement MAC (both of which leads to release of MVs). This mechanism of repair is initiated by elevated Ca^{2+}, termed as the patch hypothesis leading to stimulation of vesicle-vesicle fusion, forming a larger vesicle which is added as a patch to the PM disruption site[211].

In sea urchin the main organelle involved in sealing is the yolk granules but in somatic cells, lysosomes and enlargosomes have been proposed[209]. Lysosomes, vesicle-vesicle fusion and vesicle-PM fusion activated by calcium-regulated exocytosis is required for patch formation[213]. Furthermore, altering the morphology of lysosomes with vacuolin-1, a small molecule proposed to enlarge lysosome-derived and endosome-derived vacuoles, blocked Ca^{2+}-mediated lysosomal exocytosis, but membrane resealing continued unabated[214]. By contrast, exocytosis of enlargosomes was unaffected and so these were proposed as candidates for PM resealing[206]. However, this was not without controversy since enlargosomes, although distinct from lysosomes both morphologically and in their contents, are also widely distributed and are recruited to the PM following an increase in cytosolic Ca^{2+}[215] . Moreover, tetanus toxin, which is widely accepted as an

inhibitor of PM resealing in somatic cells, failed to abrogate exocytosis of enlargosomes[216].

It is noteworthy, that the role of enlargosomes as resealing organelles is not well characterised. Thus, the specific organelle(s) responsible for PM resealing remains unknown and so perhaps, it would be incorrect to assume that PM repair is entirely regulated by a single specialised compartment. Instead, reflecting on the involvement of an array of organelles might be a closer concept since an increase in cytosolic Ca^{2+} might also perturb a variety of local responses[217]. As aforementioned, Plasma membrane resealing is a common event in eukaryotic cells, which has fundamental biological significance in cell survival[205]. The release of MVs could be envisaged as a form of membrane damage, which leads to the rapid activation of lysosomes to the site of damage for repair.

1.10 Aims

There has recently been intense interest in the origin, transport, and the role of microvesicles in health and disease. In the present study, I investigated:

1. The possible role of MVs during cellular invasion by intracellular pathogens such as virus and if Coxsackievirus B1 (and most likely other viral pathogens) exploit a host mechanism of vesiculation to infect and spread to secondary sites of infection and cause apoptosis among infected cells.

2. To investigate whether there is a possible link between cancer drug resistance and release of MVs and why it is essential for cancer cells to stimulate the release of MVs post chemotherapy.

3. To examine if release of MVs from cells induced by anticancer drugs and agonist agents, causes damage to the integrity of the host plasma membrane before lysosomal repair.

2. Materials and Methods

2.1 Cell lines

THP-1 cells (Human promonocytic cells) were used in some experiments as the source of MVs. However, in other experiments, MVs were isolated from Jurkat cells (Human T-cells), MCF-7 cells (Human breast cancer cells) and PC3M cells (Human prostate cancer cells). HeLa cells (Human cervical cancer lines) were used for studies on Cosxackie virus B1 invasion and in some experiments, MVs isolated from these cells were utilised.

Eukaryotic cell lines: THP-1 cells (ECACC; Ref No. 88081201)

Jurkat cells (ECACC; Ref No. 88042803)

MCF-7 cells (ECACC; Ref No. 86012803)

PC3-M cells (Received as a kind gift)

HeLa cells (Received as a kind gift)

2.1.1 Cell Growth Medium (GM)

The RPMI 1640 supplemented growth medium was used to cultivate HeLa cells, PC3M, Jurkat cells, MCF-7 cells, THP-1. RPMI 1640 medium with phenol red, containing 2.05 mM glutamine and sodium pyruvate were supplemented with 10% FBS (v/v) and 1% Penicillin / Streptomycin in 500 ml volumes and stored at 4°C. MVs were removed from FBS by centrifugation before addition to RPMI.

2.2 Experimental Buffers and Solutions

2.2.1 Cell freeze medium (abcam protocol)

20%	FBS (v/v)
10%	DMSO (v/v)
1%	Pencillin/Streptomycin (v/v)
69%	RPMI

2.2.2 Virus freeze solution (original method)

95%	RPMI (v/v)
5%	FBS (v/v)

2.2.3 Lysis buffer- pH 7.4[218]

100 mM	HEPES-KOH
2 mM	$CaCl_2$
0.2%	Triton X-100 (v/v)
	Protease inhibitor (AEBSF)
	Millipore water

2.2.4 SDS-PAGE buffers

2.2.4.1 (X4) SDS Sample buffer[218]

200 mM	Tris-HCl
25%	Glycine (w/v)
2%	SDS (w/v)
0.2%	Bromophenol blue (w/v)
20 mM	DTT (added fresh on the day)
	Millipore water

2.2.4.2 (1.5 M) Resolving buffer- pH 8.8[218]

18.17 g	Tris base

Dissolved in 100 ml deionised water and pH adjusted to 8.8

2.2.4.3 (0.5 M) Stacking buffer- pH 6.8[218]

6.06 g	Tris base

Dissolved in 100 ml deionised water and pH adjusted to 6.8

2.2.4.4 Resolving gel solution (12%)[218]

2 ml	ddH_2O
1.25 ml	1.5 M Tris-HCl pH 8.8
0.05 ml	10% SDS (w/v)
1.66 ml	Acrylamide/Bis 30% (w/v)
0.025 ml	10% APS (w/v)
0.0025 ml	TEMED

2.2.4.5 Stacking gel solution[218]

1.53 ml	ddH_2O
0.625 ml	0.5 M Tris-HCl, pH 6.8
0.025 ml	10% SDS (w/v)
0.335 ml	Acrylamide/Bis 30% (w/v)
0.0125 ml	10 % APS (w/v)
0.0025 ml	TEMED

2.2.4.6 Electrophoresis running buffer (1L)[218]

30 g	Tris-HCl, pH 8.3
144 g	Glycine
50 ml	20% SDS (w/v)
950 ml	ddH_2O

2.2.4.7 Coomassie Brilliant Blue G-250[218]

0.025%	Coomassie blue (w/v)
10%	Acetic acid (v/v)
90%	ddH2O (v/v)

Mixed and filtered (Whatman number 1 paper)

2.2.4.8 Destain solution (500 ml)[218]

35 ml	Acetic acid
25 ml	Methanol
440 ml	ddH_2O

2.2.4.9 Transfer buffer (10X)[218]

250 mM	Tris base
1925 mM	Glycine
500 ml	ddH_2O

2.2.4.10 Sartoblot buffer (500 ml)[218]

40 ml	Transfer buffer (1X)
100 ml	Methanol
360 ml	ddH_2O

2.2.4.11 Ponceau solution[218]

0.25%	Ponceau S (w/v)
3%	Trichloroacetic acid (v/v)
	ddH_2O

2.2.4.12 Phosphate Buffer Saline (PBS) solution - 1L (Sigma-Aldrich)

140 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4
	ddH_2O

2.2.4.13Phosphate Buffer Saline – Tween 20 (PBS-T) (Sigma-Aldrich)

1 L	PBS
1 ml	Tween 20

2.2.4.14 Blocking buffer[218]

6%	Milk powder (w/v)
100 ml	PBS-T

2.2.4.15Antibody dilution buffer (WB)[172]

3%	Milk powder
100 ml	PBS-T

2.3 Permeabilisation Buffer (PB)[172]

0.5%	Tween 20 (v/v)
	PBS

2.4 Immunofluorescence antibody dilution buffers[172]

3%	Milk powder (w/v)
100 ml	PBS-T

2.4.1 Cell Dilution Medium[172]

10%	FBS
1%	NaN_3
	PBS solution

2.4.2 Primary and secondary antibody dilution buffer[172]

3%	BSA (w/v)
	PBS

2.4.3 Flow cytometry analysis buffer[172]

3%	BSA (w/v)
1%	NaN_3 (w/v)
	PBS

2.4.4 Veronal buffer – pH 7.4

0.15 mM	$CaCl_2$
141 mM	NaCl
0.5 mM	$MgCl_2$
1.8 mM	Na Barbital
3.1 mM	Barbituric acid

All components dissolved in 500 ml of deionised water as directed by manufacturer (Sigma-Aldrich).

2.5 Ringer's solution - pH 7.25

138 mM	NaCl
2.7 mM	KCl
1.06 mM	$MgCl_2.6H_2O$
1.8 mM	$CaCl_2$
12.4 mM	HEPES
5.6 mM	D-Glucose

All components dissolved in 500 ml deionised water as directed by manufacturer (Sigma-Aldrich).

2.6 Annexin V binding buffer – pH 7.4

10 mM	HEPES/NaoH
140 mM	NaCl
2.5 mM	$CaCl_2$

All components dissolved in 100 ml of deionised water as directed by manufacturer (Invitrogen).

2.7 Methods

2.7.1 Maintaining cell lines

2.7.1.1 Jurkat and THP-1 cell lines

Non-adherent Jurkat cells and THP-1 cells were maintained in growth medium containing RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin. In addition, cells were occasionally maintained for a week in growth medium supplemented with 1% kanamycin at 37°C in 5% CO_2 atmospheric conditions. The cells were split, depending on confluency every 3 to 5 days by washing twice with serum-free RPMI 1640. Simply, the cells were transferred into 50 ml centrifuge tubes and spun at 160 *g* for 5 min. Resulting supernatant was discarded and cells were gently resuspended in the remaining medium. Serum-free RPMI was added and cells were centrifuged as mentioned earlier. Resulting supernatant was discarded and cell pellets were resuspended in the appropriate volume of growth medium. Cells were then seeded in the desired dilution into new 75 cm^2 culture flasks. Cells were only cultured in medium supplemented with kanamycin for a week to avoid development of resistance to the antibiotic.

2.7.1.2 Adherent cell lines

Adherent cells (MCF-7, HeLa and PC3M) were also maintained at 37°C with 5% CO_2, in RPMI growth medium. These cells were also split depending on confluency every 2 to 3 days. Cells were washed twice by changing GM with serum-free RPMI and addition of 0.25% (v/v) trypsin/EDTA in RPMI. After 5 min incubation at 37°C with 5% CO_2, the flask was tapped several times to

detach the cells and growth medium was added to inactivate the trypsin. The trypsin solution was removed by centrifugation at 200 *g* for 5 min followed by one wash in serum-free RPMI. Cell pellets were resuspended in the appropriate GM volume and seeded in the desired dilution into new culture flasks. Exponentially growing cells with viability of 95% or higher were used in every experiment. The number of cells and viability were determined before the start of every experiment using the flow cytometer (ViaCount assay, Guava Technologies).

2.7.1.3 Cryopreservation of eukaryotic cells

To prepare frozen stocks for long term storage, non-adherent cells grown to almost 100% confluency were washed twice (160 *g*, 5 min) with serum-free RPMI and cell number determined using Guava viacount as mentioned in section 2.9.1 Cells were carefully resuspended in freeze mix, transferred into cryo-vials (Greiner) at $1x10^7$ cells/ml in 1 ml volumes and immediately placed on ice. The cryo-vials were frozen at –80°C in special cryo boxes, which ensure a temperature decrease of 1°C per minute. For long-term storage the deep frozen cryo-vials were transferred to liquid nitrogen cell storage tanks. Adherent cells were also frozen by a similar procedure except that cells were first trypsinised to bring them into suspension, as described earlier. Suspended cells were washed by centrifugation at 200 *g* for 5 min. Cells were resuspended in the freeze mix and transferred into cryo-vials.

2.7.2 Coxsackie virus B1 culture and freezing

Stocks of CVB1 were stored at -80°C in 2mL aliquots. This stock was diluted with 10.5mL of GM 5% FBS. Total volume of 12.5mL was then added to a confluent flask of HeLa cells and incubated with slow shaking for 1 h at room temperature. GM 5% FBS in a volume of 12.5mL was added to the flask and incubated for 16-24 h at 37°C with CO_2. CVB1 (25ml) was harvested by two freeze-thaw cycles. The medium was clarified by centrifugation at 4000rpm for 15mins (A-4-62 swing-out rotor, using 5810R centrifuge, Eppendorf). This supernatant was stored at -80°C or added to five confluent flasks of HeLa cells then the above process was repeated for production of more CVB1.

2.7.3 Cell culture

To defrost cells, cryo-vials were removed from liquid nitrogen and immediately thawed in a water-bath at 37°C. After cleaning the lid with 70% ethanol, the content was transferred to a 15 ml centrifuge tube containing 9 ml of fresh growth medium, prewarmed to 37°C and cells were sedimented by centrifugation at the appropriate speed (160 *g* or 200 *g*, 5 min for mammalian cells). To remove DMSO, the medium was discarded and the pellet was resuspended in fresh growth medium. The cells were then placed into culture flasks of the same size as had been used prior to freezing, and incubated at 37°C with 5% CO_2.

2.8 Biochemical Methods

2.8.1 Isolation of microvesicles from conditioned medium

Microvesicles were isolated by a modification of a previously described method. Conditioned medium from cells cultured with 10% FBS at 37°C and 5% CO_2, was centrifuged once at 200 *g* for 5 min to remove the cells. The supernatant was then centrifuged at 4,000 *g* for 60 min at 4°C to remove cell debris. The resultant supernatant was sonicated in a sonicating water-bath for 5 x 1min prior to centrifugation in order to disperse aggregated exosomes. Resultant supernatant was ultracentrifuged at 25,000 *g* for 2 h at 4°C. The MV pellet was resuspended in sterile PBS and quantified on a Guava EasyCyte flow cytometer using ExpressPlus software (Guava Technologies), or stained with Guava Nexin-FITC to determine surface PS expression. Isolated MVs were then used immediately or stored at -80°C for next day experiments. The above procedure is for isolation of MVs and so a slightly modified method, described below was used for isolation of more pure MVs.

2.8.2 Isolation of highly purified Microvesicles from conditioned medium

Conditioned medium from cells cultured with 10% FBS at 37°C and 5% CO_2, was centrifuged once at 200 *g* for 5 min to remove the cells. The supernatant was then centrifuged at 4,000 *g* for 1 h at 4°C to remove cell debris. The resultant supernatant was sonicated in a sonicating water-bath for 5 x 1min prior to centrifugation in order to disperse aggregated exosomes. Supernatant was then centrifuged at 25,000 *g* for 2 h at 4°C to pellet MVs.

Pelleted MVs were washed once by resuspending in sterile PBS and centrifuged again at 25,000 *g* for 2 h at 4°C to remove proteins such as albumin possibly bound to the MV membrane surface. The MV pellet was resuspended in sterile PBS and quantified, or analysed for PS exposure as described in section 2.8.1. In some experiments, MVs were isolated by reverse washing of the filter membrane, with PBS after filtration of debris-free supernatant through a 0.22 µm membrane.

2.8.3 Isolation of MVs released from stimulated cells

In some experiments cells were washed twice at 200 *g* for 5 min and preincubated at 37°C with 5% CO_2 atmospheric conditions for 1 h. Cells were centrifuged in order to remove any background MVs released during the preincubation step. Pelleted cells were resuspended in prewarmed RPMI (37°C) supplemented with 0.5 mM $CaCl_2$, and seeded into 24-well plates at $1x10^6$ cells/well in 1 ml reaction volumes. To investigate the effect of 5-FU and MTX anti-metaboilite drugs on Leukaemic cells such as THP-1 cells (human acute monocytic Leukaemia cells) and tumour cells, such as PC3M cells were treated with these drugs. In order to ascertain whether these drugs induce apoptosis in cells and whether washing the drug post treatment of the cells, aids their survival, in some experiments cells were washed after 30 min stimulation with these drugs. Cells were stimulated to microvesiculate by addition of the specific inducing agents being investigated and incubated at 37°C for 30 min with shaking.

The reaction was stopped by placing on ice for 1 min and transferred into 1.5 ml microcentrifuge tubes. Cells were pelleted by low speed centrifugation at 200 *g* for 5 min and debris removed by centrifuging at 4,000 *g* for 1 h. Importantly, samples were sonicated in a sonicating water-bath for 5 x 1 min prior to centrifugation. MVs were isolated from the resulting supernatant by centrifugation at 25,000 *g* for 2 h. This speed parameter was found to pellet MVs without much contamination with any remaining aggregated exosomes. MVs isolated were resuspended in 200 µl of PBS and quantified by flow cytometry as described in section 2.8.1.

2.8.4 Isolation of MVs by the 'reverse filtration method'

Cells and cell debris-free culture supernatants were filtered through a 0.22 µm pore size membrane filter. After filtration of the supernatant, the membrane filter was reversed and MVs bound to the surface were removed by washing with PBS. Isolated MVs were pelleted by centrifugation at 25,000 *g* for 2 h.

2.8.5 Detection of PS-Positive MVs

Isolated MVs were resuspended in Annexin binding buffer and Guava Nexin-FITC was added or not (control) in a 200 µl final volume. The sample was then incubated at RT for 30 min with shaking and centrifuged at 25,000 *g* for 2 h to pellet MVs. Samples were analysed immediately as directed by the manufacturer using the flow cytometer (Guava EasyCyte, Guava Technologies).

2.8.6 Cell treatments prior to induction of MVs

Trypsinised HeLa cells, Jurkat cells, MCF7 cells, and PC3M cells were treated with various inhibiting agents, before the induction of MVs. Cells were washed and resuspended into prewarmed, serum-free RPMI 1640 supplemented with 0.5 mM $CaCl_2$. Cells were seeded in triplicate at $1x10^6$ cells/well into12-well plates and the following agents were added at their required concentrations. Cytochalasin D (10 µM 10 min at RT), calcium ionophore (10 µM), EGTA (5mM 10 min at RT), calpeptin (20 µM 45 min at 37°C) or conA (20 µg/ml 10 min at RT) were added and incubated at indicated temperatures and times prior to induction of MVs with NHS (10%) or BzATP (300µM) for 30 min at 37°C. NHS contains MVs by itself therefore in all experiments prior to addition of NHS to the cells; MVs in NHS were removed by ultracentrifugation.

The above experiment was repeated for HeLa, using CVB1 as the inducing agent. In some experiments, semiconfluent HeLa cells were plated at $5X10^4$ cells/well in GM into 12-well plates containing sterilized 18-mm round coverslips and incubated overnight at 37°C, 5% CO_2. Cells were washed the next day with serum-free RPMI and pre-treated with the agents mentioned earlier. In addition, cells were induced to microvesiculate with inducing agents and the reaction was incubated at 37°C for 30 min with shaking.

2.8.6.1 Cell treatment prior to plasma membrane repair assays

To investigate the role of MVs in damage repair mechanisms, trypsinised HeLa cells, MCF7 cells and PC3M cells were preincubated with concanavalin A (conA), 20 µg/ml for 10 min at RT and washed once by centrifugation at 200 *g* for 5 min. Cells were also incubated with the inhibiting agents for 45 min prior to addition of MTX, Doc and 5-FU. All chemical agents were washed-off prior to addition of drugs. Cells were incubated with the drugs for 30 min at 37°C with 5% CO_2. Cells were fixed with 4% paraformaldehyde and were labelled with anti-LAMP-1 AlexaFluor-488 as described under section 2.11. Labelled cells were analysed by flow cytometry using the ExpressPlus assay. In some experiments, semiconfluent HeLa cells treated as described above were examined by fluorescence microscopy for detection of secreted lysosomes.

2.8.6.2 Cell treatments prior to infection experiments

HeLa cells were seeded into 12-well plates and incubated overnight prior to performing infection experiments. On the day of the experiment, culture medium was changed twice with serum-free RPMI 1640 and preincubated with various agents at the required concentrations. Semiconfluent HeLa cells were preincubated with the inhibiting agent calpeptin and cells were washed once by changing medium with fresh serum-free RPMI prior to addition of CVB1.

2.8.7 Quantification of intracellular calcium

To measure intracellular calcium and LAMP-1 expression in membrane resealing, HeLa cells ($5x10^5$/well in triplicate) were trypsinised and washed twice with phenol Red-free RPMI. Cells were then resuspended in Ringer's solution with 2mM $CaCl_2$ containing Calcium Green-1AM (5µM) in microcentrifuge tubes. Cells were left unstimulated (NI) or stimulated to microvesiculate with inducing agents, after preincubation with calpeptin (20µM at 37°C, 45 min), conA (20µg/ml, at RT, 10min) or EGTA (5mM). Cells were immediately fixed with 4% PFA (10 min, at RT) and washed 1x with cold PBS. Samples were labelled with LAMP-1-Alexafluor 488 (5µg/ml). Labelling was performed in 3% BSA/PBS/NaN_3 at 4°C for 1h with shaking. Samples were washed 3x with cold PBS and resuspended with 3% BSA and analyzed by flow cytometry. In addition, supernatant after stimulating with BzATP was purified and released MVs were analyzed. Similar experiments were performed using 10% NHS as the stimulus.

2.8.8 Protein Quantification

The concentration of a protein solution was determined using the BCA Protein Assay Kit (Pierce, Thermo Scientific, UK). It uses a combination of the biuret reaction (reduction of Cu^2+ ions to Cu+ ions by proteins in an alkaline medium) and the colorimetric detection of the Cu+ - cations by a bicinchoninic acid-containing colour reagent. Following the manufacturer's instructions, 10 µl of protein samples were diluted in double distilled water

(ddH2O; dilution 1:5) and added to 200 µl working solution consisting of a mixture of kit reagent A and reagent B (ratio 50:1 respectively).

In parallel a dilution series of a 0.5 µg/µl BSA stock solution in ddH_2O was prepared and used as standard in later evaluation. Applied concentrations were 0, 62.5, 125, 250, 500, 750, 1,000, 1,500 and 2,000 µg/ml BSA in 500 µl ddH_2O. Volumes equal to samples were added to 200 µl of a mixture of kit reagent A and B (ratio 50:1) in a 96 well plate followed by incubation at 37°C for 30 min. After incubation at room temperature for 10 min to cool samples, A_{540} readings were taken on a FLUOstar Omega microplate reader. Protein concentrations of the unknown samples were determined by interpolation on a standard curve multiplied by a dilution of factor of 5.

2.8.9 Preparation of cell lysates/ protein extraction

Cells ($1x10^7$) grown in culture flasks were sedimented by centrifugation (160 *g*, 5 min at 15°C). The pellet was washed once by careful resuspension in RPMI followed by centrifugation. After that, cells were counted using a haemocytometer and lysis was performed to give a protein concentration equivalent to $2x10^6$ cells/10 µl. If defined amounts of protein were required, the pellet was lysed with detergent based lysis followed by determination of total protein concentration using the described procedure for the BCA kit. Briefly, cell lysis was performed by resuspension of the pellet in 0.2% Triton X-100 (w/v) containing protease inhibitor cocktail. To solubilise membrane proteins, samples were repeatedly pipetted and insoluble materials were

sedimented by centrifugation (5,000 rpm, 5 min at 4°C, A-4-62 swing-out rotor, using 5810R centrifuge, Eppendorf). The total protein concentration was determined of the resultant supernatant and then subjected to SDS-PAGE analysis.

2.8.10 Sample preparation for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS sample buffer (4X) was added to samples in a ratio of 1:4 followed by incubation at 95°C for 4 min. Before loading the samples onto the gel a centrifugation step was performed (2,000 *g*, 2 min) to collect all liquid at the bottom of the reaction microtube and loaded into the gel wells.

2.8.11 SDS-PAGE Protein Molecular Weight Standards

As a protein molecular weight standard, prestained Protein-Marker I (BioRad) was used. Prestained markers, ranging from 10 to 194 kD or 10 to 250 kD were used when analysing gels by Western blot using the ECL detection system. Markers were applied by loading 10 µl into wells.

2.8.12 SDS-Polyacrylamide Gel Electrophoresis

To separate proteins, which were denatured by sodium dodecyl sulphate (SDS) according to their molecular masses, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Laemmli, 1970) using the Mini PROTEAN III Electrophoresis System (Bio-Rad)[218]. Gels with dimensions of 102 x 73 mm and a thickness of 0.75 mm were cast between

two glass plates by pouring freshly prepared 12% separating gel solution containing acrylamide/bisacrylamide into the gel cassette fixed in a casting frame. Unpolymerized separating gel solution was overlaid with H_2O-saturated butanol to achieve an even surface. After polymerization, H_2O-saturated butanol was poured off, washed twice with deionised water, and the excess water was blotted using a filter paper (Whatman 3 MM, Whatman AG). Then unpolymerized stacking gel was poured into the gel cassette and a plastic comb was inserted from the top, to form the loading wells in the stacking gel. After polymerization, the gels were used immediately.

To perform electrophoresis, the gel was placed into the electrode assembly device inside a clamping frame in the tank of the Mini PROTEAN III system. Electrophoresis running buffer was added to the inner and outer chambers of the tank and the plastic comb was carefully removed. Wells were washed to remove any free unpolymerized acrylamide/bisacrylamide with the running buffer. Samples were loaded into the wells of the stacking gel using extra long loading pipette tips. Electrophoretic separation was performed at 150 V (constant voltage) until the bromophenol blue front of the SDS sample buffer reached the end of the resolving gel. Gels were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membrane for Western blotting analysis.

2.8.13 Western Blotting Assay

Proteins separated by SDS-PAGE were transferred to a Hybond C nitrocellulose membrane for further analysis using a semidry transfer device (Bio-Rad Sartoblot system). A Hybond C nitrocellulose membrane and two pieces of blotting paper (Whatman 3 MM, BioRad) were cut to the size of the separating gel. Blotting paper, nitrocellulose membrane and the sandwich-blotting cassette were equilibrated in Sartoblot buffer. One piece of blotting paper was placed on the cathode plate, and the nitrocellulose membrane was placed on top of the blotting paper. The gel was removed from between the sandwich-blotting cassette and placed on top of the membrane, and a second blotting paper was also placed on top of the gel. Having removed air bubbles, the anode plate, also dampened with the Sartoblot buffer was used to complete the sandwich. Electroblotting was carried at 15 V (constant voltage) for 1 h[218].

2.8.14 Reversible Ponceau Staining of Proteins

To verify successful protein transfer, hybond C nitrocellulose membranes were reversibly stained with Ponceau S (Serva Electrophoresis GmbH). The nitrocellulose membrane was incubated in Ponceau solution for 1 min. Subsequently excessive Ponceau solution was washed away using deionised water until the protein bands became clearly visible. Marker bands were labelled using a ball pen and the membrane was then completely destained[218].

2.8.15 Protein Detection using the ECL Kit

Western blotting was performed as described above using hybond C nitrocellulose membrane (Amersham Biosciences). The membrane was incubated in blocking buffer for 1 h at room temperature or at 4°C, overnight, on a shaker. Following blocking, the membrane was rinsed with PBS-T and incubated with the primary antibody in the desired dilution for 1 h at room temperature on a shaker. Six 10 min washing steps with PBS-T were performed and the membrane was incubated with secondary goat anti rabbit IgG or goat anti mouse IgG antibodies coupled to HRP in a 1:5000 dilution. After six 10 min washes with PBS-T on a shaker, visualization was performed using the enhanced chemiluminescence reagent system (ECL, Amersham Pharmacia). The ECL solutions (reagent A and B) were mixed in equal volumes and the membrane was incubated with the mixture for 1 min at room temperature and chemiluminescence was detected using a UVP ChemiDoc-It system (UVP systems, UK).

2.9 Flow cytometry

The Guava Easy Cyte flow cytometer allows complex biological studies such as cell counting and viability testing, white blood cell phenotyping, cytokine detection, cell activation marker analysis and other complex molecular analyses to be performed simultaneously. This saves time, but also generates accurate results, which comes much closer to mimicking the complex biological responses within the body. The Guava flow cytometer can be used to perform ten different assays; however, for these studies only three

assays were implemented. These were the ViaCount assay (for counting cells and determining viability), ExpressPlus assay (for cell activation marker analysis, cytokine expression, microvesicle analysis and studies on protein-protein interaction) and Nexin assay (for reporting apoptosis).

2.9.1 Cell counting and Guava ViaCount

Cell number and viability were determined using the ViaCount assay, which distinguishes between viable and non-viable cells based on the differential permeabilities of two DNA-binding dyes in the Guava ViaCount reagent. The nuclear dye only stains nucleated cells, while the viability dye brightly stains dying or dead cells. This proprietary combination of dyes enables the Guava ViaCount assay to distinguish viable, apoptotic and dead cells. Cell debris is excluded from results based on negative staining with the nuclear dye.

2.9.2 Cell culture and Virus labelling

All the cell lines were maintained at 37°C in 5% CO_2 humidified conditions. Jurkat and HeLa cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete growthmedium, GM). In addition, cells were occasionally maintained for a week in GM supplemented with 1% kanamycin. Exponentially growing cells were counted and viability was determined using the Guava ViaCount Reagent by flow cytometry (ViaCount assay; Millipore). Throughout, after three days in culture, cells were split 1:4 and only cultures with at least 95% viability were used in experiments.

Coxsackievirus B1 (CVB1) (Batch No: NCPV 231) was obtained from the Health Protection Agency (HPA, UK) and expanded by infecting HeLa cells. CVB1 was purified through centrifugation and titres were determined by plaque assay on HeLa cells. For infection, cells were inoculated with CVB1 at a multiplicity of 50 PFU/cell and kept at 37°C until fixation for flow cytometry or immunofluorescence assays were performed.

Mouse anti-CVB1 monoclonal antibody (mAb) was obtained from Millipore (Billerica, MA). Flow cytometry analysis of cell associated viral proteins was carried out with anti-CVB1 monoclonal antibody followed by Alexafluor 488-conjugated anti-mouse IgG (Life Technologies Ltd, Paisley, UK) referred together as anti-CVB1 Alexafluor 488 antibody.

2.9.3 Cell labelling

HeLa cells were labelled with Annexin V AlexaFluor 488, at RT for 15 min with shaking. Annexin V AlexaFluor 488 was diluted in a binding buffer provided with the kit and used at 1 μg/10^6 cells, according to the manufacturer's instructions. Cells were washed three times with serum-free RPMI prior to experiments.

In some experiments, semiconfluent HeLa cells were labelled with anti-LAMP-1 AlexaFluor 488 (5μg/ml) at 4°C for 1 h with shaking. Labelled cells were washed three times with serum-free RPMI prior to use in experiments. To study membrane repair mechanisms, HeLa cells were also labelled with

50 µg/ml of propidium iodide (PI) at RT for 1 min prior to fixation with 4% paraformaldehyde. For phalloidin staining, HeLa cells were washed 2x with prewarmed PBS and fixed with 4% PFA for 10 min at RT. Samples were washed 2x with PBS and permeabilised with 0.5% Triton X-100/PBS at RT for 5min. Samples were washed again (2x) with PBS and preincubated with 1% BSA/PBS for 20min and stained with 20µl of phalloidin in 200µl of PBS per coverslip and incubated at RT for 20min or at 4°C for 1h with shaking. Cells were washed 2x with PBS and mounted on slides for microscopy.

2.9.4 Immunofluorescence staining for cytokines

In order to determine the expression of cell activation markers as well as surface and intracellular cytokines by specific antibody staining, cells were processed according to the following protocol and analysed using flow cytometry. Cells were harvested, washed by centrifugation at 200 *g* for 5 min and total cell number and viability (usually >95%) was determined using ViaCount reagent (refer to 2.9.1). Cells were usually stained in eppendorf tubes and later transferred into a 96-well microtiter plate for analysis on the flow cytometer; however, they can also be stained in polystyrene round-bottom, 12 x 75 mm Falcon tubes.

Cell surface expression of Fas was analysed by flow cytometry (EasyCyte, Guava Technologies, UK). Jurkat cells (2 x 10^6) were washed twice (200 *g*, 5 min each) with PBS and resuspended in cold PBS containing 10% FBS and 1% NaN_3. Cells were incubated in the dark with primary antibodies (10

µg/million cells, R&D systems, UK) against tested molecules at 4°C for 1 h with shaking. After three washes (400 *g*, 5 min), cells were stained with the isotype-matched controls (anti-mouse or anti-rabbit IgG-FITC, R&D systems) diluted 1:320 in PBS with 3% BSA and incubated with shaking, in the dark, at 4°C for 1 h. Cells were again washed three times with cold PBS, resuspended in 200 µl of PBS containing 3% BSA, 1% NaN_3 and analysed immediately using flow cytometry and the ExpressPlus assay program.

For staining of intracellular molecules, Jurkat cells ($2x10^6$/ reaction) in triplicate were resuspended in PB (0.5% Tween 20 in PBS) at room temperature for 20 min. Cells were permeabilised by washing three times with PB and incubated with 2 µg of primary antibodies at 4°C for 1 h in PBS with 10% FBS/ 1% NaN_3. Cells were washed three times and incubated with IgG-FITC-labelled secondary antibodies (4°C, 1 h), and again washed three times with PBS. Samples were resuspended in 200 µl each of PBS containing 3% BSA, 1% NaN_3 and analysed immediately by flow cytometry.

2.9.5 Immunofluorescence followed by Induction of MVs

To further investigate the notion that MVs carry molecules from the cell upon release, surface and intracellular immunostaining experiments were repeated in duplicate. Briefly, $1x10^7$ cells/reaction were washed as mentioned earlier and resuspended in PBS containing 10% FBS only. For intracellular staining, cells were first permeabilised with PB at RT for 20 min. Cells were labelled with primary (10 µg/reaction) and secondary antibodies (1/320 dilution)

according to steps described earlier. Antibody-labelled cells were resuspended in prewarmed PBS supplemented with 2 mM $CaCl_2$ and stimulated to microvesiculate by addition of 10% NHS. After incubation at 37°C for 30 min, MVs were isolated as described under section 2.8.2. Purified MVs were analysed on the Guava EasyCyte flow cytometer for cytokines using appropriate antibodies and IgG-FITC as negative control.

2.9.6 Induction and purification of apoptotic Microvesicles (aMVs) or virus-induced apoptotic MVs (vaMVs)

Conditioned medium from Jurkat cells not stimulated (healthy) or stimulated to generate apoptotic MVs (aMVs) using cyclohexamide 1 mg/ml (CHX) or dexamethasone (DEX) 1 mM (both from Sigma-Aldrich) at 37°C for 5 h or 3 h respectively, were first centrifuged at 200 *g* for 5 min to pellet cells. The supernatant was centrifuged at 4,000 *g* for 1 h to remove cell debris, and then sonicated for 5 min in a sonicating water-bath (Townson and Mercer Ltd, Croydon) to disperse aggregated exosomes. The resultant supernatant was then centrifuged at 25,000 *g* for 2 h to pellet MVs. Pelleted MVs were resuspended in sterile PBS and washed once by further centrifugation at 25,000 *g* for 2 h. Finally isolated MVs were resuspended in sterile PBS and quantified using a flow cytometer (ExpressPlus, EasyCyte, Millipore).

For virus induction of MVs from cells, semiconfluent HeLa cells seeded in 12-well plates were infected with CVB1 (50 PFU/cell) at 37°C for 4 h. Cells were then washed once with RPMI 1640, resuspended in complete medium and

incubated at 37°C, 5% CO_2 for 48 h. Conditioned medium from infected cells was collected and MVs were isolated and quantified as described above.

2.9.7 *In vitro* apoptosis assay

HeLa cells ($5x10^4$ cells/well) were seeded in triplicate in 12-well plates containing 18 mm coverslips, washed twice with serum-free RPMI 1640 and resuspended in GM. Semiconfluent cells were co-cultured with aMVs derived from DEX or CHX-induced Jurkat cells at a 1:1 or 5:1 (aMVs to cell) ratio in triplicate. Plates were incubated at 37°C for 30 min, 2 h, 5 h and 24 h. Apoptosis was determined by staining cells with Guava ViaCount Reagent (ViaCount assay, Guava Technologies) and analysed by flow cytometry. To determine the role of Fas-Fas ligand complex, as well caspases, semiconfluent HeLa cells seeded overnight in 12-well plates were washed and pretreated with anti-Fas or anti-FasL antibodies (5 µg/ml), or preincubated with the pan-caspase inhibitor, ZVKD-fmk (50 µM) at 37°C for 30 min. Without washing, cells were co-cultured with aMVs (5:1) and incubated at 37°C for 5 h. Apoptosis was determined by staining cells with Guava ViaCount Reagent and analysed by flow cytometry.

For studying apoptosis induced by CVB1 or virus-induced apoptotic MVs (vaMVs), semiconfluent HeLa cells were pretreated or not with calpeptin (20 µM) at 37°C for 45 min and washed prior to addition of CVB1 or vaMVs. HeLa cells were inoculated with CVB1 using 50 PFU/cell (throughout) or vaMVs at 5:1 (vaMVs to cell) ratio and incubated at 37°C for 48 h.

Throughout CVB1 infection experiments were first incubated at 37°C for 4 h, washed to remove extracellular virions and further incubated at 37°C for 48 h in complete medium. Apoptosis was determined by staining with Guava ViaCount Reagent and analysed by flow cytometry.

For studying apoptosis induced by anticancer drugs or drug-bearing MVs, semiconfluent HeLa, MCF7 or PC3M cells were pretreated or not with calpeptin (20 μM) at 37°C for 45 min and washed prior to addition of various concentrations of anti cancer drugs, or addition of drug-bearing MVs. Cells treated with drugs were washed with serum free RPMI after 30 min and resuspended in GM and incubated at 37°C with 5% CO_2 for 72 h. Apoptosis was determined by staining with Guava ViaCount Reagent and analysed by flow cytometry every day. Cells were co-cultured with drug-induced MVs incubated at 37°C with 5% CO_2 for 48 h and apoptosis was determined by staining with Guava ViaCount Reagent and analysed by flow cytometry every day.

2.9.8 Knockdown of *CAPNS1* by small interfering RNA (siRNA) transfection

GeneSolution siRNA sequences targeted to four different sites in *CAPNS1* mRNA (GeneBank) and negative control siRNA (Qiagen, Crawley, UK) were reconstituted in sterile RNase-free water at a final concentration of 10 μM. For CVB1 infection experiments, HeLa cells ($5x10^4$/well in triplicate) were transfected with 50 nM siRNA, using HiPerfect transfection reagent (HPP,

Qiagen) for 48 h prior to performing experiments. The sequence for the human *CAPNS1* siRNAs were: siRNA#1, 5' -CAC CTG AAT GAG CAT CTC TAT -3'; siRNA#3, 5' -AAG GTG GCA GGC CAT ATA CAA -3'; siRNA#5, 5' -CAG CGC CAC AGA ACT CAT GAA -3'; siRNA#6, 5' -TCC GAC GCT ACT CAG ATG AAA -3'. Negative control siRNA, 5' -AAT TCT CCG AAC GTG TCA CGT -3'. Consistent reduction of *CAPNS1* expression was observed with siRNA#6, and so siRNA#6 was used to assess the effects of decreasing *CAPNS1* levels on the sensitivity of PC3M cells to drug resistance and HeLa cells to CVB1 induction of vaMVs and apoptosis.

2.9.9 Immunoblotting analysis of siRNA transfected cells

Control or CAPNS1 knocked down HeLa cells, PC3M cells, or purified MVs were lysed with lysis buffer (100 mM HEPES/KOH, 2 mM $CaCl_2$, 0.5% Triton X-100) containing cocktail protease inhibitor (Sigma-Aldrich). The protein concentration of lysates was measured using the BCA assay kit (Pierce Biosciences) and 20 µg was resolved by SDS-PAGE on a 12% acrylamide gel. Immunoblotting was carried out by transferring proteins to nitrocellulose membrane (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK) at 100 mA, constant current for 1 h using the semidry transfer system (BioRad). Blots were blocked overnight at 4°C in 6% non-fat milk dissolved in PBS, and then incubated with the murine monoclonal antibodies anti-CVB1 (Millipore), anti-β-actin (Sigma-Aldrich), anti-calpain small subunit 1 (*CAPNS1*) (Sigma-Aldrich), or rabbit anti-caspase-3 antibody (abcam) diluted 1/500 in PBST (PBS with 0.1% (v/v) Tween 20).

Blots were washed six times in PBST for 10 min each time, and where required, membranes were then probed with the secondary antibodies, anti-mouse-HRP or anti-rabbit-HRP (1/1000). After 1 h at room temperature with shaking, blots were washed six times in PBST for 10 min each. Protein bands were visualised using the LumiGOLD ECL Western Blotting Detection kit (SignaGen Laboratories, Rockville, MD 20850), and the chemiluminescence signal detected using ChemiDoc-It Imaging System (UVP, LLC, Cambridge, UK).

2.9.10 Immunofluorescence analysis of infected cells

For flow cytometry analysis, HeLa cells ($5x10^4$/well) seeded overnight in 12-well plates were washed twice with sterile PBS and inoculated with CVB1 in 50 PFU/cell. Plates were incubated at 37°C for 4 h, and then washed twice with sterile PBS and incubated at 37°C for 48 h. Cells were trypsinized after 48 h, fixed and then permeabilised using the Fix and Perm Cell Permeabilisation kit (ADG, Germany) according to the manufacturer's instructions. During permeabilisation, cells were incubated with anti-CVB1 antibody (5 µg/ml) at room temperature (RT) for 30 min shaking in the dark. Cells were washed three times (400 *g*, 5 min) with cold PBS and stained with mouse anti-IgG-FITC (1/200) resuspended in PBS with 3% BSA for 30 min at RT with shaking. After washing three times with PBS, cells were analyzed by flow cytometry to determine intracellular viruses.

For fluorescence microscopy, HeLa cells were seeded overnight in 12-well plates containing 18 mm coverslips. Semiconfluent cells were inoculated with CVB1 in 50 PFU/cell and incubated at 37°C for 4 h, and then 48 h. Cells were fixed and permeabilised as described above and labelled at RT for 30 min with anti-CVB1 antibody (10 µg/ml) diluted in 3% BSA/PBS. After washing three times with PBS, cells were resuspended in 3% BSA with PBS containing Alexafluor 488 anti-IgG antibody (1/200), and incubated at RT for 1 h with shaking. Plates were washed three times and coverslips were inverted on to microscopic slides using DAPI-Vectashield (Vector Laboratories, Burlingame, CA). Images were captured using a fluorescent microscope (IX81 motorized inverted fluorescent microscope, Olympus).

2.9.11 Quantification of cytokines by ELISA

MVs were isolated from Jurkat cells stimulated with DEX or CHX at 37°C for 5 h or 3 h respectively. The concentrations of Fas and caspase-3 were measured by using 10 µg proteins in ELISA kits (R&D Systems) according to the manufacturer's instructions, and absorbance readings were measured using the FLUOstar Omega plate reader (BMG Labtech, UK).

2.10 Quantification of FM1-43 dye in membrane resealing

Semiconfluent HeLa or PC3M cells seeded on 12-well plates were placed in Ringer's solution containing 2mM $CaCl_2$ and FM1-43 dye (10µg/ml). Cells were preincubated with or without conA 20µg/ml at room temperature for 10 min with shaking, and then stimulated with various agents such as MTX, Doc,

and EGTA. Relative FM1-43 fluorescence was measured over 30 min on the FLUOstar Omega plate reader (BMG Labtech, UK).

2.11 Quantification of LAMP-1 and Calcium Green-1AM fluorescence

HeLa cells ($5x10^5$/well in triplicate) were trypsinised, washed and resuspended in Ringer's solution with 2mM $CaCl_2$ containing Calcium Green-1AM (5μM) in microcentrifuge tubes. Cells were left unstimulated (NI) or stimulated (at 37°C, 30min) to microvesiculate with BzATP (300μM), after preincubation with calp (20μM), conA (20μg/ml, RT, 10min) or EGTA (5mM). Samples were immediately fixed with 4% PFA (10 min, RT) and washed 1x with cold PBS. Samples were labelled with LAMP-1-Alexafluor 488 (5μg/ml). Labelling was performed in 3% BSA/PBS/NaN_3 at 4°C for 1 h with shaking. Samples were washed 3x with cold PBS and resuspended with 3% BSA and analyzed by flow cytometry. In addition, supernatant after stimulating with BzATP was purified and released MVs were analyzed. Similar experiments were performed using 10% NHS as the stimulus.

2.12 Fluorescence microscopy

For fluorescent microscopy analysis, adherent cell samples were placed into 12-well plates containing 18mm coverslips cultured on coverslips at 37°C with 5% CO_2 for 24 h. Cells were treated as mentioned in the section 2.11. Cells then were gently washed twice with PBS at the final stage and mounted on microscope slides with DAPI-VECTASHIELD medium (Vector Laboratories Inc. Burlingame, CA) for fixed cells and mounting medium (Agar

Scientific, Essex, UK) for fixed MVs. Coverslips were mounted on microscope slides and images were collected using fluorescence microscope (1X81 motorized inverted fluorescence microscope, Olympus Corporation).

2.13 Electron microscopy

2.13.1Transmission electron microscopy

All processing steps were carried out in a fume cupboard, as hazardous chemicals were being used. Cells ($5x10^6$/ml, usually > 95% viability) were washed twice with RPMI and resuspended in fresh prewarmed (37°C) RPMI supplemented with 0.5 mM $CaCl_2$, were either stimulated (DEX, 1 mM) or not (control), and fixed with 0.1 M fixative solution (3% glutaraldehyde in 0.1 M sodium cacodylate buffer [pH 7.2]). Fixed samples were incubated at 0°C for 1 h in 1% osmium tetroxide solution (2% osmium tetroxide (Sigma-Aldrich), 1:1, 0.2 M sodium cacodylate buffer) and block stained by resuspending in 1% aqueous uranyl acetate (overnight on a rocker). Samples were dehydrated in an ascending ethanol series (from 70% to 100% absolute ethanol, v/v, 30 min each time) after resuspending in 1% hot agarose, and washed twice (30 min each) with propylene oxide (Agar Scientific, Essex, UK). Dehydrated samples were infiltrated with a 1:1 mixture of propylene oxide: Agar resins (mixture of 4.8 g agar resin, 3.6 g MNA, 1.9 g DDSA and 0.2 g BDMA, Agar Scientific) and incubated overnight at room temperature with shaking. Capsule-embedded samples polymerized at 60°C for 24 h, were cut on a Leica Ultracut R ultra microtome (Leica Microsystems, Deerfield, IL) and stained for 10 minutes in Reynolds lead citrate stain[219]. The

ultra thin sections were then examined on a Jeol JEM – 1200 Ex II electron microscope (JOEL, Peabody, MA).

2.13.2 Negative staining

Pure isolated MV samples for Negative staining were also taken to the EM Unit at the LSHTM and stained with 2% aqueous uranyl acetate or 2% PTA (phosphotungstic acid) pH 6.8 + aqueous Bacitracin (300 µg/ ml diluted 1:10 in the negative stain acts as a spreading agent). Using fine tipped Watchmaker forceps to handle the 400 mesh copper grids with a pioloform support film (Grids and Pioloform powder from Agar Scientific Limited, Essex CM24 8DA), the grids were pre-treated with 1% aqueous Alcian Blue 8GX for 10 minutes before rinsing in MilliQ water. 5 µl of the sample (MVs) were placed on the grids for 1 min. This was then removed by touching the grid edge with a strip of filter paper and replaced with 5 µl of the stain for a further 1 min. The stain was then removed in the same way and the grid was allowed to air-dry, before examination on the Transmission Electron Microscope. Digital images were recorded using the AMT digital camera previously described for the examination of stained ultrathin sections.

2.14 Determination of viral titre by plaque assay

Virus stock was tittered by means of a plaque assay on HeLa cells and stored at -80°C. HeLa cells ($5x10^4$cells/well) were seeded into 12-well plates and incubated at 37°C, 5% humidified CO_2 for 24 h. Cells were washed twice with RPMI and resuspended in GM. CVB1 stock was serially diluted in

10-fold steps and added to the confluent cells monolayer. Cells were incubated at 37°C, 5% humidified CO_2 for 4 h. During incubation, agarose overly medium was prepared by dissolving 2% agarose in 50 ml GM using microwave. Cells washed twice with RPMI and were overlayed with GM containing 2% agar when the temperature reached 40°C. When the agarose had set, plates were incubated at 37°C, 5% humidified CO_2 for 5 days. Overlay medium was removed carefully and cells were resuspended in 500µl of 4% PFA and incubated for 15 min at RT for fixation. Cells were washed with PBS. Cells were then stained with 0.2% of crystal violet for 1 h. Finally, crystal violet was removed and cells were washed twice with tap water. The plaques were counted by microscope, and the viral concentration was calculated as PFU per millilitre (**Fig.2.1**). The first image is indicative of negative control cells without any viral infection and in the second image the arrow is indicative of formation of one plaque.

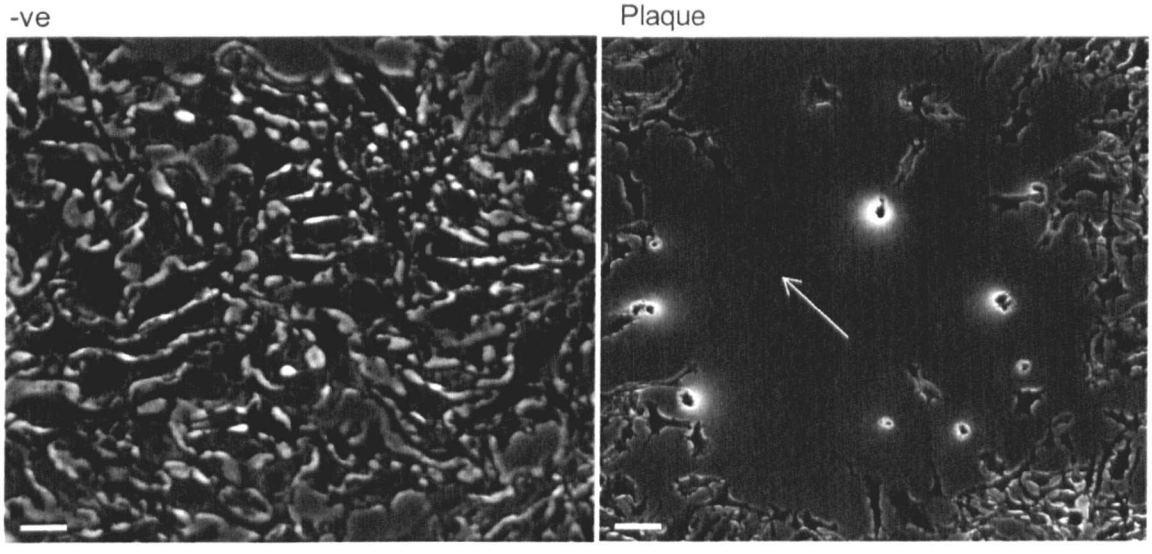


Figure 2.1 The titre of Coxsackie virus was determined on monolayers of HeLa cells by Plaque assay. HeLa cells ($5X10^4$ cells/well) were seeded in into 12 wells plate and incubated at 37°C, 5% humidified CO_2 for 24h. Cells were washed twice with RPMI and resuspended in GM with 5% FBS. Multiple samples of CVB1 were serially diluted in 10-fold dilutions and overlaid on 95-100% cells and left to adsorb at 37°C, 5% humidified CO_2 for 4 h. Cells were washed twice with RPMI and an overlay of GM with 2% agar was added and incubated at 37°C, 5% humidified CO_2 for 5 days. Overlay medium then was removed carefully and cells were resuspended in 500µl of 4% PFA and incubated for 15 min at RT for fixation. Cells were washed with PBS. Cells were then stained with 0.2% of crystal violet for 1h. Finally, crystal violet was removed and cells were washed twice with tap water. The plaques were counted, and the viral concentration was calculated as PFU per milliliter. The first image is indicative of negative control cells without any viral infection and in the second image the arrow is indicative of formation of one plaque. All assays were performed in triplicates. Scale bare = 200 µm.

2.15 Statistical Data Analysis

Statistical analysis for all data presented was performed by the unpaired *t*-test for repeated measures using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). Differences giving a value of $P < 0.05$ with confidence interval of 95% were considered statistically significant.

3. CVB1-induction of apoptotic MVs reveals a nonlytic MV-to-cell mechanism of virus transmission to new cells

3.1 Introduction

Coxsackievirus B1 (CVB1) is an enterovirus of the *Picornaviridae* family, which are among the most common human pathogens. CVB1 is a non-enveloped, single-stranded RNA virus associated with a broad spectrum of human diseases including myocarditis, meningoencephalitis, pancreatitis and paralytic myelitis[139, 141]. It was recently suggested that progression of viral infections mainly result from the interaction between virulence factors and the host immune system. This interplay activates inflammatory responses that ultimately contribute to tissue damage[220]. Moreover, it is generally accepted that enteroviruses, like many other non-enveloped viruses have to induce lysis of host cells in order to spread infection[221]. Thus upon infection with CVB1, the fate of infected host cells mainly depends on the induction of apoptotic (programmed cell death) or non-apoptotic (necrosis and autophagy) cell death[143].

Apoptosis is a programmed cell death, which occurs after sufficient cellular damage. This damage correlates with a distinct set of biochemical and physical changes involving the nucleus, cytoplasm and the plasma membrane. Cells round up (in the process losing contact with neighbouring cells) and shrink during the early stages of apoptosis. In the cytoplasm, ER dilates causing the cisternae to swell and this forms vesicles and vacuoles. Nuclear chromatin starts to condense and aggregate into dense compact masses, which are degraded in the nucleus by endonucleases. In this process, phosphatidylserine is translocated from the inner to the outer

membrane leaflet and degraded nucleus becomes convoluted and buds off into several fragments which are referred to as apoptotic bodies[222].

By contrast, necrosis is characterised by irreversible swelling of the cytoplasm, organelles and ultimate lysis of the plasma membrane. Necrosis results in the release of cytoplasmic materials including degradative enzymes from the cell into the surrounding area that can trigger cellular damage to neighbouring non-necrotic cells[197, 198]. Earlier reports have suggested that CVB1-induced apoptosis is a viral mechanism to facilitate maximum virus dissemination[144, 151]. While it remains unclear the exact process(s) involved, some studies have hinted that CVB1 induction of apoptosis in neighbouring cells is not exclusively caused by the lytic escape of enteroviruses[152, 153]. In agreement, earlier studies have postulated a direct cell-to-cell spread of the poliovirus in the central nervous system[154]. Moreover, a recent study has also described a nonlytic viral mechanism of cell-to-cell transmission that involves CVB3 induction of cellular protrusions[130].

Earlier reports which showed that picornaviruses disrupt the host cytoskeleton early during their infection[147,223], and also to facilitate virus release[146], and recent studies which showed that disruption of the host cytoskeleton results in release of microvesicles prompted us to investigate the role of these vesicles in the spread of CVB1-induced apoptosis. As reviewed before, microvesicles (MVs) also known as microparticles[224], plasma membrane-derived vesicles[225] or ectosomes[226], are small intact

membrane vesicles released constitutively from cells or upon activation with extracellular stimuli. An increase in host cell intracellular calcium ($[Ca^{2+}]_i$) levels leads to activation of the enzyme, calpain and depolymerization of the actin cytoskeleton resulting in release of the vesicles. Recent reports by us have showed that MVs participate in intercellular communications by carrying numerous cytokines and acute-phase proteins from the parental cell[172].

Others have shown that MVs can act as vehicles of immunosuppression[177], participating in cancer progression[70] and inflammatory responses[227]. In a recent report, monocyte-derived MVs were shown to deliver a cell death message via encapsulated caspase-1 to vascular smooth muscle cells (VSMCs)[97]. In addition, we recently reported a novel *T. cruzi* cell invasion strategy that involves parasite utilisation of host cell vesicle release[172]. We showed that *T. cruzi*-elicited release of MVs results in damage to the integrity of the host plasma membrane, which the parasite then exploits for entry before lysosomal repair.

Here I show that CVB1 requires activation of calpain and disruption of the host cytoskeleton via MV release for infection. Upon entry, CVB1 causes further release of vaMVs from infected cells, which carry death signals including caspase-3 that can be delivered to healthy viable cells rendering them apoptotic. I further show that vaMVs also carry CVB1 virions which can induce apoptosis in target cells, and thus propose a nonlytic MV-to-cell virus spread mechanism by which non-enveloped viruses rapidly disseminate to neighbouring cells to cause infection.

We have already described the transfer of TGF-β1 and other leaderless cytokines via membrane fusion of MVs to target cells during differentiation of monocytes to macrophages[172]. Moreover, we have recently reported that fusion between host cell-derived MVs and *T. cruzi* accelerates decay of the C3-convertase, thus conferring protection of the parasite against complement-mediated lysis[228]. Such transmission via MV, in the case of a non-enveloped virus could be envisioned to provide an essential advantage for the virus by helping it to hide inside the MV to avoid immune defense by host neutralising antibodies. In addition, MVs being important entities during intercellular communications[226], could aid distant dissemination of viruses and further infections without activating the host immune system. Together, these findings indicate that CVB1 (and most likely other viral pathogens) exploit a host mechanism of vesiculation to infect and spread to secondary sites of infection and cause apoptosis among infected cells.

3.2 Results

3.2.1Dexamethasone and cyclohexamide-induced Jurkat cells, release apoptotic MVs and express Fas. In previous work, CMIRC and others have shown that MVs carry signalling molecules which could be delivered to target cells[69, 172]. In the present study, I wanted to ascertain whether cells undergoing apoptosis release apoptotic MVs (aMVs), which carry death signals that could be transferred to healthy target cells. I first stimulated Jurkat cells with DEX (1 mM) or CHX (1 mg/ml), or left non-stimulated (control), and incubated at 37°C for 5 h. Cells were then fixed and processed

for transmission electron microscopy analysis, or in other experiments, released MVs were isolated by differential centrifugation and analysed by flow cytometry. I was able to confirm the typical dot plot distribution of MVs as assessed by the logarithmic amplification of forward light scatter (FSC) and side light scatter (SSC) signals (**Fig.3.1a**). Unstimulated cells displayed a smooth plasma membrane (PM) (**Fig.3.1b**, left panel), as opposed to DEX-induced cells, which had a rough PM and released MVs (**Fig.3.1b**, right panel). To test whether DEX or CHX induces apoptosis in cells, Jurkat cells were stimulated with the agonists and incubated at 37°C for 1 h or 5 h. Cells were stained with Guava Nexin Reagent and checked for annexin V (AnV) binding to PS exposed on the outer membrane leaflet (a hallmark of apoptosis). Compared to control (non-induced), stimulated cells were predominantly at early apoptosis (AnV^+ only) after 1 h incubation, but entered late apoptosis (AnV^+/7-AAD^+) after 5 h (**Fig.3.1c**). In addition, DEX-induced cells expressed higher surface Fas (52%), compared to CHX (27%) and non-induced cells (6%)(**Fig.3.1d**) after labelling with mouse anti-Fas-FITC antibody.

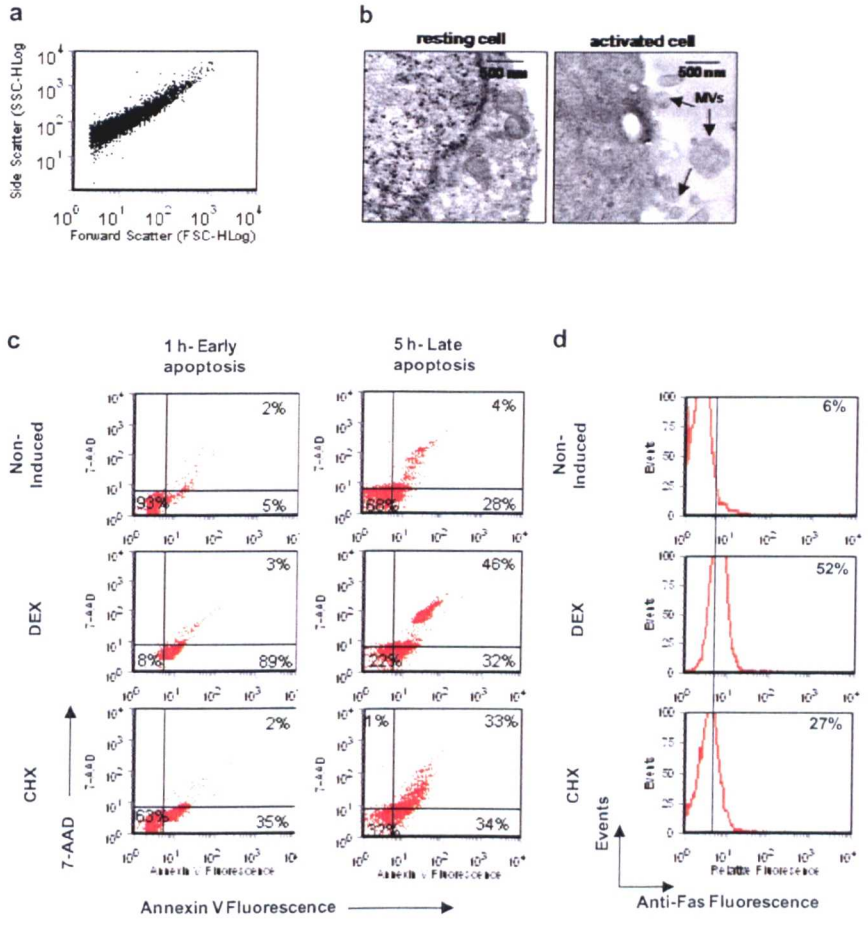


Figure 3.1 Apoptotic MVs (aMVs) released by DEX and CHX-induced Jurkat cells carry Fas. (**a**) Cells release microvesicles upon stimulation with external agonists. Jurkat cells ($1x10^6$/well in triplicate) were stimulated at 37°C for 5 h with dexamethasone (1 mM) or cyclohexamide (not shown). Released aMVs were isolated and quantified by flow cytometry based on forward scatter (FSC) and side scatter (SSC) signals as described in materials and methods. (**b**) Transmission electron microscopy of Jurkat cells releasing aMVs after stimulation or not with DEX at 37°C for 5 h, and immediately fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Resting cells show a smooth plasma membrane as opposed to activated cells, which show the production of aMVs. (**c**) Flow cytometry analysis of activated cells in apoptosis. Jurkat cells ($1x10^6$/well in triplicate) were left unstimulated, or activated with dexamethasone (DEX, 1 mM) or cyclohexamide (CHX, 1 mg/ml) at 37°C for 1 h or 5 h. After 1 h incubation with DEX, approx 89% of cells were in early apoptosis as opposed to non-induced and CHX-treated cells where only 5% and 35% respectively entered early apoptosis. However, more cells entered late apoptosis after 5 h incubation with the agonists. (**d**) Flow cytometry analysis show Jurkat cells express surface Fas after stimulation for 1 h with DEX (52%) or CHX (27%). Data are representative of results from several experiments.

3.2.2 Microvesicular release of Fas and caspase-3 by DEX and CHX-induced Jurkat cells. As it was observed surface expression of Fas on Jurkat cells by flow cytometry analysis (**Fig.3.1d**), and detected presence of the receptor in conditioned medium from these cells (not shown), I wondered whether aMVs also contained Fas and other death molecules. This is because MV production has been reported previously as a mode of cytokine release into the extracellular microenvironment[73, 172, 229]. MVs isolated after DEX and CHX stimulation, or MVs from unstimulated Jurkat cells were analysed for the presence of Fas by ELISA. The vesicles were either lysed or left unlysed to ascertain the location of the receptor. 'Fas' was detected in MVs isolated from non-induced and CHX-induced cells, but was the highest in DEX-stimulated MVs (**Fig.3.2a**).

Strikingly, higher levels of Fas were detected in DEX-induced MVs after lysing the vesicles; by contrast, lower amounts of the molecule were measured in MV-free supernatant (**Fig.3.2a**). This implies that soluble 'Fas' is predominantly carried on the surface membrane, or encapsulated within MVs. In addition, higher levels of caspase-3 were measured in apoptotic MVs as opposed to vesicles obtained from healthy cells (**Fig.3.2b**).

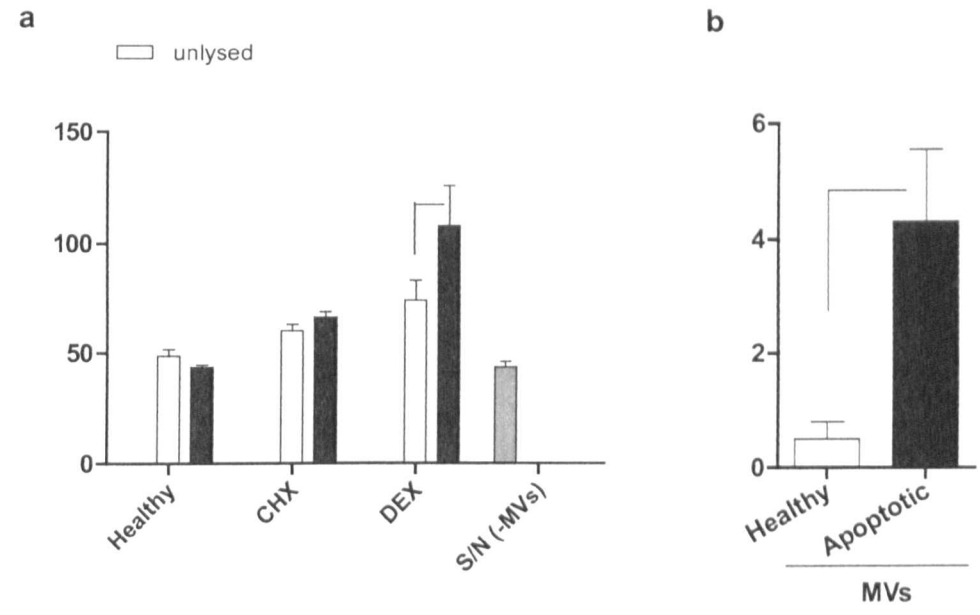


Figure 3.2 Agonist-induced aMVs carry encapsulated Fas and caspase-3 molecules. (**a, b**) ELISA analysis shows presence of Fas and caspase-3 molecules in apoptotic MVs. Jurkat cells ($1x10^6$/well in triplicate) were left inactivated, or stimulated with DEX (1 mM) and CHX (1 mg/ml) at 37°C for 5 h. Apoptotic MVs as well as healthy MVs isolated and analysed by flow cytometer, were lysed or not and protein levels were quantified using the BCA kit on a plate reader. MV lysates (10 µg) as well as MV-free culture supernatants were loaded on to an ELISA kit (R&D Systems) according to the manufacturer's protocol and assayed for Fas (**a**) or caspase-3 (**b**). Data represents the mean ± SD of two separate experiments performed in triplicate. $^*P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 were considered statistically significant.

3.2.3 DEX and CHX-induced apoptotic MVs deliver death molecules to healthy target cells. To determine whether aMVs could deliver the death molecules to healthy cells and thus render them apoptotic, DEX and CHX-induced MVs were isolated from Jurkat cells and analysed by flow cytometry. HeLa cells ($5x10^4$/well in triplicate) were co-cultured with the apoptotic MVs isolated from conditioned medium of either unstimulated Jurkat cells or cells induced with DEX or CHX at 1:1 or 5:1 (MVs to cell) ratio. Experiment was incubated at various times and apoptosis was assessed using Guava Nexin Reagent by flow cytometry. Apoptotic MVs co-cultured with cells as early as 30 min induced approximately 10% death of HeLa cells as compared to control healthy vesicles (5%), (**Fig.3.3a**). Interestingly, CHX-induced MVs caused greater apoptosis (20%) at 30 min when added at 5:1 (MVs to cell) ratio. The lower apoptosis levels observed with DEX could only be due to the fact that 30 min incubation is not enough time for the agonist to induce effective cell death (**Fig.3.3a**). Nonetheless, significant levels of apoptosis were observed after 2 h co-culture of apoptotic MVs with healthy cells (**Fig.3.3b**), and this reached approximately 50% for DEX and CHX-induced aMVs after 5 h incubation (**Fig.3.3c**). No further increase in apoptosis was observed after 24 h co-culture of aMVs with healthy HeLa cells (**Fig.3.3d**). In order to test the involvement of encapsulated caspase-3 or Fas inside vesicles in this cell death induction, HeLa cells were co-cultured with Jurkat cell-derived aMVs at 5:1 (MVs to cell) ratio in presence of mouse anti-FasL or anti-Fas antibodies and the pan-caspase inhibitor, ZVKD-fmk. Experiments were incubated at 37°C, 5% humidified CO_2 and MV-induced apoptosis was

measured after 5 h. Indeed, all antibodies were capable of inhibiting aMV-induced cell death without causing further apoptosis (**Fig.3.3e and f**). Treatment with the pan-caspase inhibitor, ZVKD-fmk, reduced HeLa cell death by approximately 67%. Interestingly, anti-FasL and anti-Fas antibodies reduced DEX-induced aMV apoptosis by approximately 70% and 86% respectively (**Fig.3.3e**). Similarly, anti-FasL and anti-Fas antibodies prevented approximately 79% and 88% of HeLa cell death induced by CHX-triggered aMVs respectively (**Fig.3.3f**).

Moreover in the presence of the pan-caspase inhibitor, ZVKD-fmk, apoptosis triggered by CHX-induced aMVs was reduced by approximately 55%. It should be noted that the percentage of apoptosis values reported were compared to the levels stimulated by control healthy MVs isolated from viable Jurkat cells.

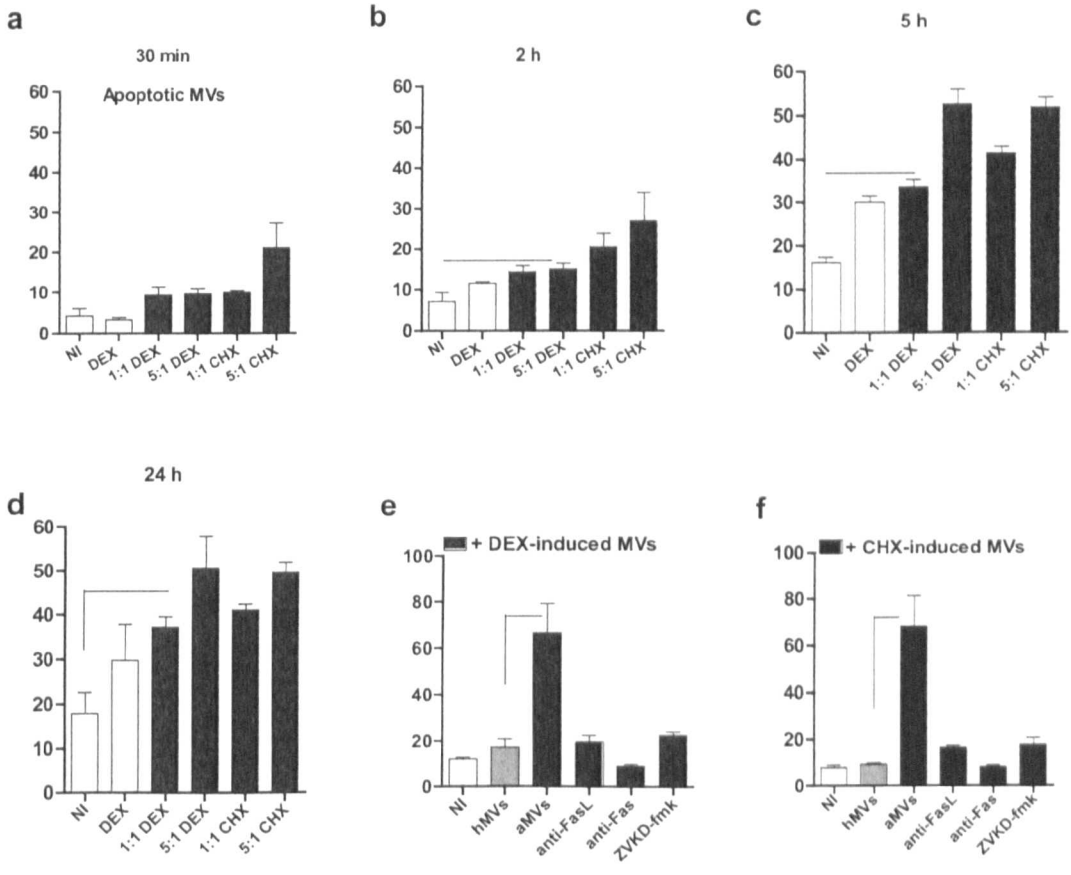


Figure 3.3 Agonist-elicited aMVs transfer death molecules to new recipient cells. (**a-d**) Semiconfluent HeLa cells were co-cultured with apoptotic MVs isolated from DEX or CHX-induced Jurkat cells at 1:1 or 5:1 (MVs to cell) ratio at 37°C, 5% CO_2 at various times. After each incubation time, HeLa cells were assayed by flow cytometry to detect apoptosis. Cells left non-induced (NI) or stimulated with dexamethasone (DEX) were used as controls. Apoptosis in recipient HeLa cells was analysed by flow cytometry using Guava ViaCount Reagent (Millipore) after 30 min (**a**), 2 h (**b**), 5 h (**c**) and 24 h (**d**). (**e and f**) Inhibition of Fas and caspase activities reduces aMV induction of apoptosis in target cells. HeLa cells were either co-cultured with healthy MVs (hMVs) or aMVs isolated from DEX (**e**) and CHX-induced Jurkat cells (**f**) in presence or absence of mouse anti-FasL (5 µg/ml), anti-Fas (5 µg/ml) or the pan-caspase inhibitor, ZVKD-fmk (50 µM), and incubated at 37°C, 5% CO_2 for 24 h. Data presented is the mean ± SD of three independent experiments performed in triplicate. **P* <0.05, ***P* <0.01, ****P* <0.001 were considered statistically significant.

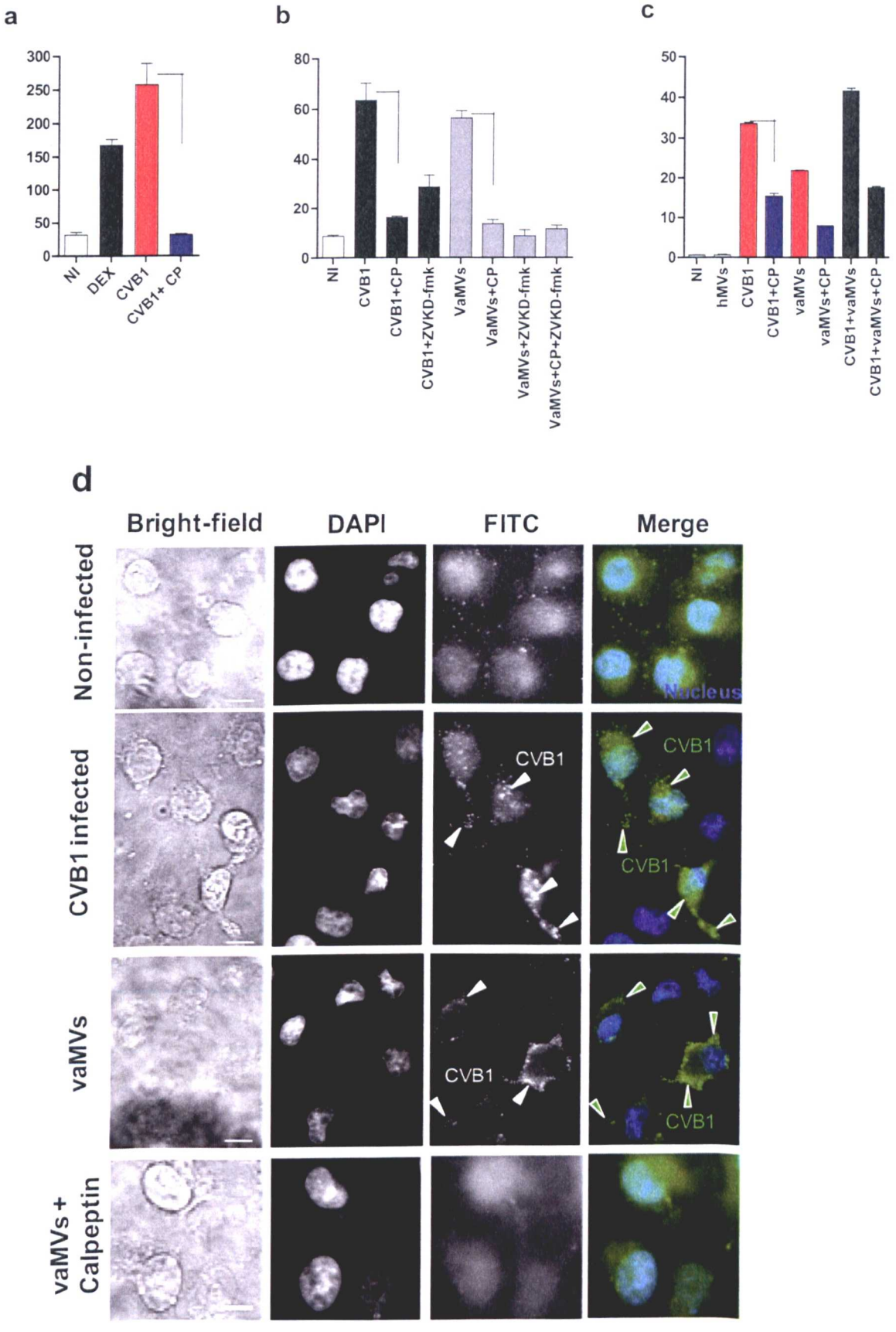
3.2.4 Virus-induced apoptotic MVs, deliver active encapsulated death molecules to healthy target cells. During infection with CVBs, the virus induces apoptosis in the infected host cells to facilitate release of viral progeny, which subsequently infects neighbouring cells. However, the enhanced levels of apoptosis measured during CVB infection cannot always be attributed to direct induction by lytic viruses[153]. As such, I first examined the possibility that CVB1 stimulates vaMVs from HeLa cells, and that these vaMVs carry death molecules including caspase-3 as for aMVs derived from DEX and CHX-induced cells. HeLa cells ($5x10^4$/well in triplicate) were infected with CVB1 (50:1 PFU/cell) at 37°C, 5% CO_2 for 48 h after pretreatment (37°C, 45 min) or not with the calpain inhibitor, calpeptin (20 μM). As for agonist-induced cells, CVB1-infected HeLa cells also released significant numbers of MVs, which was abrogated to control (Non-induced) levels after pretreatment with calpeptin (**Fig.3.4a**).

Further studies showed that these vaMVs could induce apoptosis in healthy cells after 48 h incubation, and like CVB1-induced apoptosis, vaMV-induced cell death can also be reduced after pretreating recipient cells with calpeptin (**Fig.3.4b**). Moreover, CVB1 as well as vaMV induction of apoptosis in healthy cells was significantly reduced in the presence of pan-caspase inhibitor, ZVKD-fmk, and further synergistic reduction was observed when the antibody and calpeptin were added concomitantly (**Fig.3.4b**).

The reduction of vaMV-elicited apoptosis by calpeptin (**Fig.3.4b**) prompted me to investigate whether vaMVs carry CVB1 virions. This is because I have earlier showed that inhibition of calpain activity with calpeptin prevents damage to the integrity of the host cytoskeleton, which most intracellular pathogens otherwise exploit for entry[172]. I reasoned that upon CVB1 infection, infected cells must stain positively for anti-CVB1-antibody conjugated to Alexafluor 488 anti-IgG antibody to indicate intracellular viruses. However if virus infection depends on depolymerization of the host cytoskeleton, then calpeptin-treated cells would show less staining for intracellular CVB1. I hypothesized that cells co-cultured with vaMVs would stain positive for anti-CVB1-Alexafluor fluorescence, if vaMVs carry active virus particles that could be delivered to neighbouring cells.

To investigate, semiconfluent HeLa cells were left untreated (NI), or co-cultured with healthy MVs or CVB1 particles, or vaMVs after pretreatment with calpeptin at 37°C for 48 h. CVB1-infected cells were first incubated at 37°C, 5% CO_2 for 4 h, washed and then incubated for 48 h. Cells were washed, gently trypsinized and fixed and permeabilised (Fix and Perm Cell Permeabilisation kit, ADG, Germany). Flow cytometry analysis using mouse anti-CVB1-Alexafluor antibody, confirmed the presence of virus particles inside infected HeLa cells after co-incubation with CVB1 (~33%), or vaMVs (~23%) (**Fig.3.4c** – red bars).

Moreover, less anti-CVB1 staining was detected in cells pretreated with calpeptin prior to co-culturing with CVB1 (15%) or vaMVs (8%) (**Fig.3.4c** – blue bars). In contrast, no anti-CVB1-Alexafluor fluorescence was detected in cells left untreated, or cells co-cultured with healthy MVs (**Fig.3.4c** – white bars). Of note, cells co-infected with CVB1 and vaMVs stained significantly for anti-CVB1-Alexafluor fluorescence (~42%) (**Fig.4.4c**). Furthermore, immunofluorescence imaging confirmed the presence of CVB1 virions in vaMV-infected cells, and this is reduced after calpeptin treatment (**Fig.3.4d**).To further ascertain the presence of CVB1 as well as death molecules inside vaMVs, 20 µg of proteins obtained from healthy or apoptotic lysates of HeLa MVs and cells, were resolved by a 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and immunoblotted with the murine antibodies, anti-CVB1 and anti-β-actin, or rabbit anti-caspase-3 antibody (**Fig.3.4e**). Together, these data imply that CVB1 requires calpain activation for infection and subsequent induction of vaMVs. Released vaMVs carry encapsulated death molecules, as well as CVB1 virions, thus acting as a non-lytic mechanism by which CVB1 reaches new cells to cause infection at secondary sites.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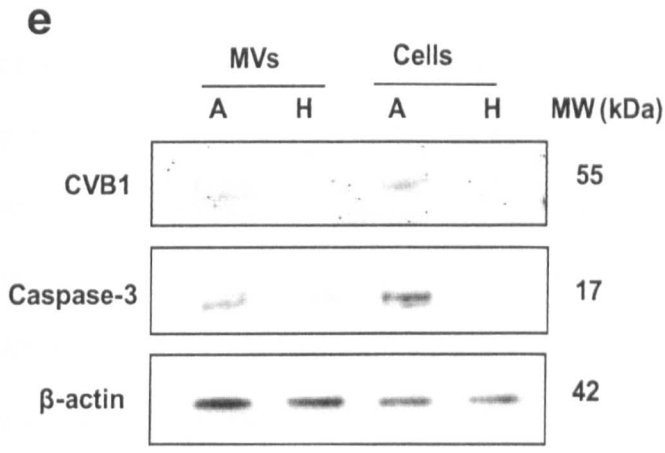

Figure 3.4 CVB1 induces release of vaMVs that carry encapsulated virions, which can infect and elicit apoptosis in new cells. (**a**) Flow cytometry analysis of MV production after semiconfluent HeLa cells were infected with CVB1 in presence or absence of calpeptin (CP) (20 μ M, 37°C for 45 min) for 48 h. (**b**) HeLa cell apoptosis induced by CVB1 particles (50 PFU/cell) or virus-induced apoptotic MVs (vaMVs, 5:1), both in presence or absence of calpeptin was assayed using the Guava Nexin Reagent (Millipore) and analysed by flow cytometry. (**c**) Flow cytometry analysis of intracellular CVB1 fluorescence after co-culture of CVB1 particles with HeLa cells, or co-incubation of various MVs (healthy and apoptotic) with cells. Cells were pretreated with calpeptin prior to addition of CVB1 particles or vaMVs and incubated at 37°C for 48 h. Data presented is the mean ± SD of three independent experiments performed in triplicate. $^{**}P$ <0.01, $^{***}P$ <0.001 were considered statistically significant using Student's *t*-test. (**d**) Fluorescence microscopy images of semiconfluent HeLa cells left non-infected or incubated with either CVB1 or vaMVs in presence of absence of calpeptin for 48 h. Cells were fixed and permeabilised prior to labelling with mouse anti-CVB1 Alexafluor 488 antibody. Images were collected using an Olympus IX81 inverted fluorescence microscope (Olympus Corp, Germany). Scale bar= 20 μm. Representative images of duplicate experiments. (**e**) Western blotting analysis of HeLa cell or MV lysates isolated from healthy or apoptotic cells infected with CVB1. Proteins were resolved on a 12% SDS-gel and transferred to nitrocellulose membrane. Membrane was immunoblotted using the mouse antibodies anti-CVB1 (1/500) and anti-β-actin (1/500), or rabbit anti-caspase-3 antibody (1/500).

3.2.5 Virus-induced MVs carry CVB1 virions which elicit further production of vaMVs and cause apoptosis. Given the presence of CVB1 virions inside vaMVs, I sought to determine whether these apoptotic vesicles would elicit production of more vaMVs. This could offer an explanation to the role of vaMVs in the rapid dissemination of CVB1 (likely other virus pathogens) to neighbouring cells. I co-cultured HeLa cells ($1x10^5$/well in triplicate) with healthy MVs, 10:1 (hMVs to cell) ratio or vaMV isolated from CVB1-infected HeLa cells in a dose response. As shown (**Fig.3.5a** – white bars), as much for non-induced cells, hMVs failed to induce further production of MVs; similar to non-induced cells. Conversely, vaMVs significantly stimulated release of more MVs, albeit not in a dose response manner (**Fig.3.5a** – coloured bars). Moreover, unlike DEX-induced apoptotic MVs which stimulated production of more aMVs without being affected by calpeptin, vaMV-elicited release of MVs was significantly reduced when cells were pretreated with calpeptin (**Fig.3.5b**). Further analysis confirmed the presence of apoptosis in HeLa cells co-cultured with vaMVs, and this was reduced after pretreatment with calpeptin (**Fig.3.5c**); also observed by microscopy (**Fig.3.5d**). As expected, hMVs failed to induce apoptosis in target cells. Together, these data further confirm the presence of CVB1 virions inside vaMVs, and like the lytic counterpart, show that CVB1 encapsulated within vaMVs also require calpain activation and depolymerization of the host cytoskeleton for infection and enhancement of vaMV production, which then aids the dissemination of virus particles to new cells in a nonlytic MV-to-cell mechanism.

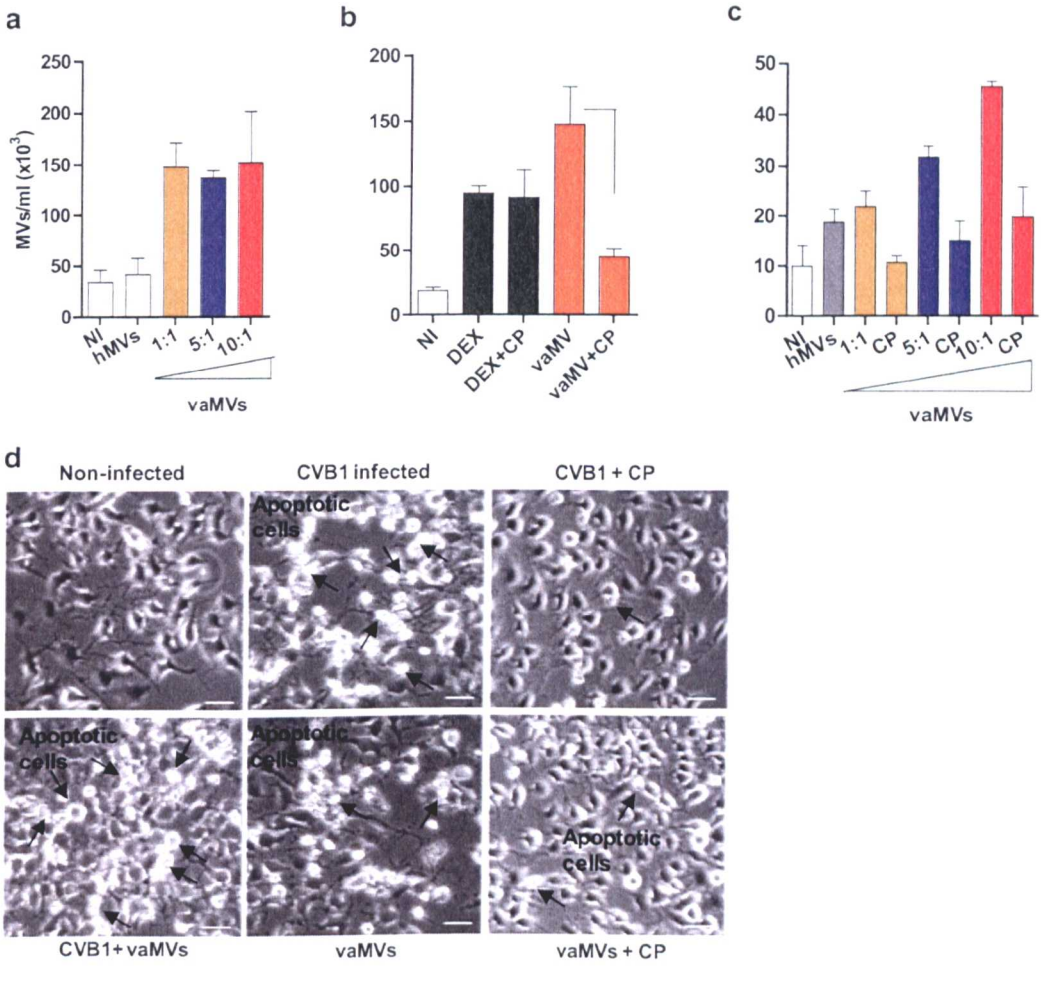

Figure 3.5 vaMVs elicit release of more vaMVs that induce apoptosis in target cells. (**a**) Flow cytometry analysis of HeLa cells left untreated or co-cultured with healthy MVs (hMVs) or virus-induced apoptotic MVs (vaMVs) at increasing amounts for 48 h. (**b**) Semiconfluent HeLa cells left non-induced (NI) or stimulated with dexamethasone (DEX, 1 mM) or vaMVs 5:1 (vaMVs to cells) ratio in presence or absence of calpeptin were assayed at 37°C for 48 h. Released MVs were isolated by centrifugation and analysed by flow cytometry. (**c**) Flow cytometry analysis of apoptosis in HeLa cells left untreated or co-cultured with healthy MVs (hMVs) or HeLa cell-derived vaMVs in presence or absence of calpeptin (CP) at 37°C for 48 h. Apoptosis was assessed by staining with Guava Nexin Reagent according to the manufacturer's instructions. Data represents the mean ± SD of two separate experiments performed in triplicate. $^{**}P$ <0.01, $^{***}P$ <0.001 were considered statistically significant. (**d**) Microscopy images of HeLa cells infected or not with CVB1 or vaMVs in presence or absence of calpeptin. Scale bar= 200 µm. Images are representative of duplicate experiments performed in triplicate.

3.2.6 Downregulation of calpain expression inhibits CVB1 entry and virus-elicited apoptosis in HeLa cells. Previous reports have showed that calpain inhibition protects against virus-induced myocardial cell death[153], and prevents release of virus progeny post-infection[222]. Some reports have also described an increase in calpain activity prior to reovirus-induced apoptosis[230],whilst others have speculated on the role of the cytoskeleton during host cell infection by CVBs[231].

Interestingly, pretreatment of HeLa cells with calpeptin significantly abrogated CVB1-induced apoptosis, as well as vaMV-elicited cell death (**Fig.3.4b**). Similarly, calpeptin treatment reduced CVB1 infection of HeLa cells as assessed by the low anti-CVB1-Alexafluor fluorescence by flow cytometry analysis (**Fig.3.4c** and **d**). To properly understand the role of calpain during CVB1 infection and subsequent induction of vaMVs from infected cells, as well as during virus-triggered apoptosis in target cells, I performed infection assays on siRNA treated HeLa cells. I carried out a knockdown of both μ- and m-calpain isoforms using calpain small-subunit 1 small interfering RNA (*CAPNSI siRNA*). Effectiveness of silencing was assessed by Western blotting (**Fig.3.6a**), by the disappearance of a 28 kD band, by flow cytometry analysis (**Fig.3.6b** and **c**) and immunofluorescence microscopy (**Fig.3.6d**).

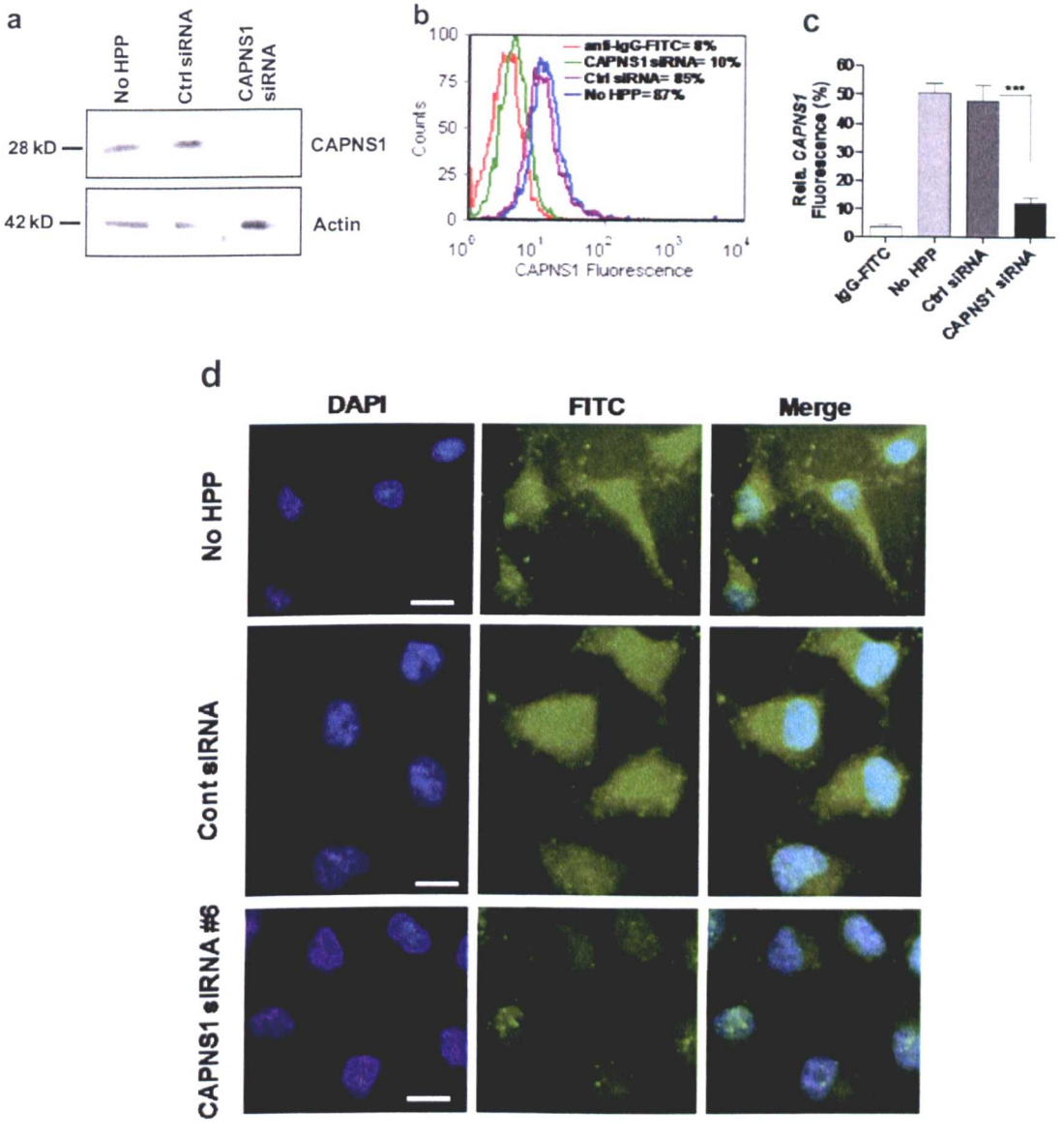

Figure 3.6 Assessement of downregulation of calpain expression by various methods. (**a-d**) Semiconfluent HeLa cells were non-transfected (No HiPerfect transfection reagent (HPP) or transfected with 50 nM of control (ctrl) siRNA or various *CAPNS1* siRNA sequences targeting different regions for 48 h. Effect knockdown was assessed by Western blotting by resolving 30 µg of protein using SDS-PAGE and transferring to a nitrocellulose membrane (**a**). (**b and c**) Flow cytometry analysis of *CAPNS1* knockdown in siRNA treated cells. (**d**) Immunostaining of CAPNS1 in HeLa cells transfected or not with control siRNA or *CAPNS1* siRNA for 48 h, as analysed by fluorescent microscopy. Scale bar= 20 µm. Data presented is the mean ± SD of three separate experiments performed in triplicate. $^{**}P$ <0.01, $^{***}P$ <0.001 were considered statistically significant using Student's *t*-test.

3.2.7. Effect of calpain silencing on vaMV production and CVB1 infection. Semiconfluent HeLa cells were non-transfected (No HiPerfect transfection reagent (HPP) or transfected with 50 nM of control (ctrl) siRNA or various *CAPNS1* siRNA sequences targeting different regions for 48 h. Knockdown of *CAPNS1* led to a noticeable decrease in CVB1 induction of MV release (**Fig.3.7a**) as opposed to untreated (HPP) or control siRNA treated HeLa cells. Moreover, silencing of *CAPNS1* significantly reduced CVB1 induction of apoptosis in target cells (**Fig.3.7b**), thus together confirming the role of calpain in MV production and virus-elicited apoptosis. Since calpain knockdown results in abrogation of MV release, I sought to confirm the importance of MV production in CVB1 infection and spread of apoptosis. As shown in **Fig3.7c** and **d**, decreased CVB1 infection was observed in *CAPNS1* knockdown HeLa cells. Notably, knockdown of *CAPNS1* which leads to abrogation of vaMV production, resulted in a dramatic reduction of CVB1 infection as assessed by the low anti-CVB1 fluorescence (from 33% to 17%) after flow cytometry (**Fig.3.7c**) and immunofluorescence microscopy analysis (**Fig. 3.7d**).

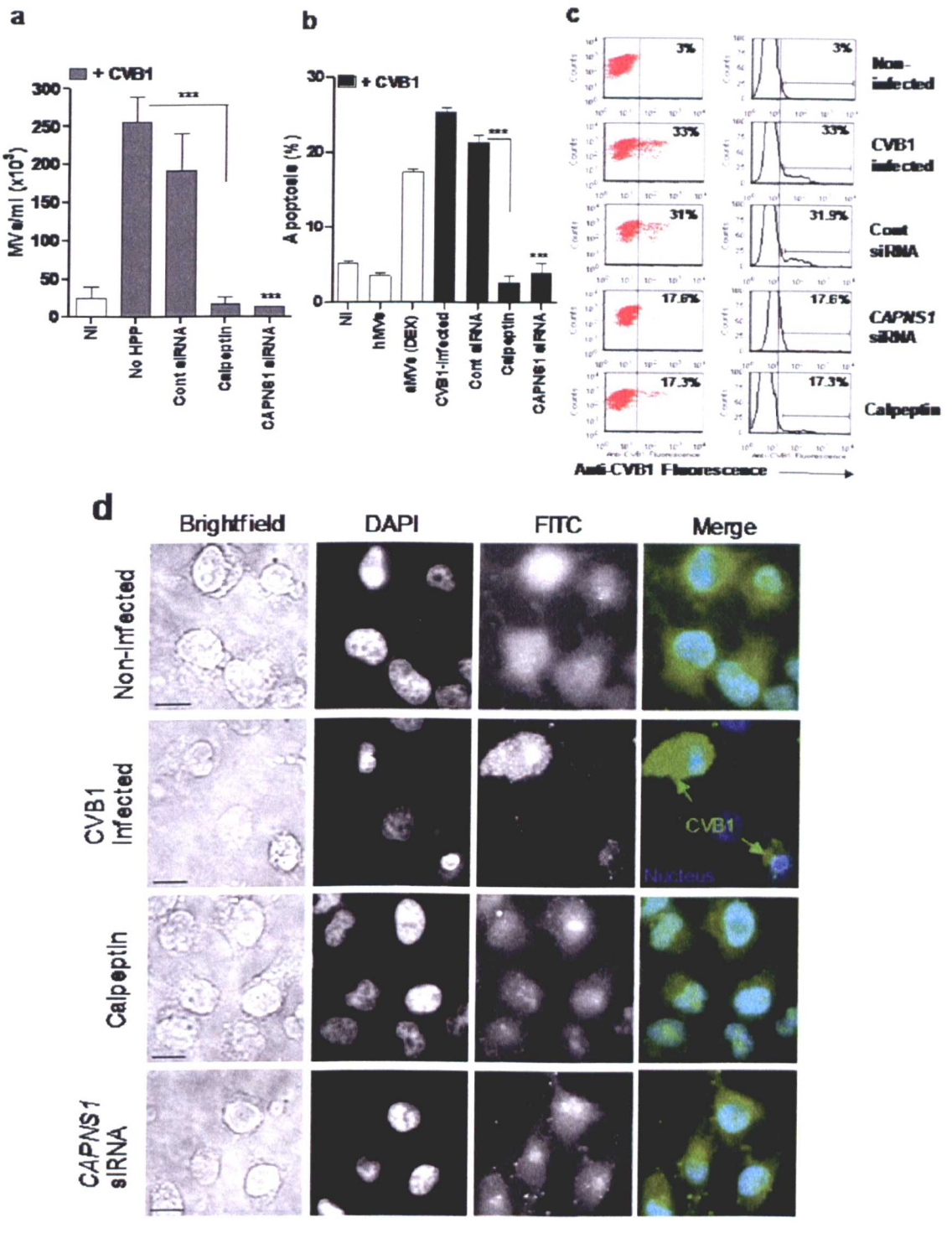

Figure 3.7 Effect of calpain silencing on vaMV production and CVB1 infection. (**a-d**) Semiconfluent HeLa cells were non-transfected or transfected with siRNA or various *CAPNS1* siRNA sequences targeting different regions for 48 h.(**a**) Untreated HeLa cells or siRNA-treated cells were co-cultured with CVB1 (50 PFU/cell) for 48 h and released vaMVs were isolated and quantified by flow cytometry. (**b**) Apoptosis in siRNA-treated HeLa cells infected or not with CVB1 were assessed by staining with Guava ViaCount Reagent and analysed by flow cytometry. (**c**) Flow cytometry analysis of intracellular CVB1 after siRNA treatment of HeLa cells. Representative data of two independent experiments performed in triplicate. (**d**) Immunostaining of CVB1 inside infected HeLa cells after siRNA treatment for 48 h, as assessed by fluorescent microscopy. Scale bar = 20 µm.

3.3 Discussion

Many infectious pathogens have developed strategies to subvert host epithelia or endothelial barriers in order to invade and spread to secondary sites of infection. Recent studies have established that CVBs enter polarized cells in an endocytic mechanism that requires the activation of specific signalling molecules including the Src family of tyrosine kinases[142, 232]. Although non-enveloped viruses such as reoviruses have been shown to cause damage through direct effect of viral infection of myocardiocytes[153], it is well established that the enhanced spread of viral progeny to secondary sites during infection is not exclusively achieved via the lytic mechanism of infection[152, 153]. While it remains unclear the exact mechanisms involved, a recent study demonstrated a nonlytic cell-to-cell strategy that involves CVB3 induction of cellular protrusions[130]. But in spite of such corroborative study, to date very few data supporting a nonlytic viral spread mechanism has been accrued. Furthermore, although these data suggest a nonlytic cell-to-cell mechanism, it still remains unclear how viral progeny disseminate to more distant regions to infect new cells.

Here I describe for the first time, a nonlytic mechanism of infection involving MVs, by which CVB1 virions encapsulated within vaMVs are disseminated to secondary sites to infect new cells. Microvesicle release is an important host mechanism by which cells intercommunicate with each other. For example, platelets release MVs which transfer tissue factor to monocytes, thus allowing monocytes to participate in the coagulation pathway[176]. In addition,

CMIRC reported that MVs could act as an unconventional secretory pathway, by which molecules lacking an N-terminal sequence could be transported to the extracellular matrix[172]. Despite these essential functions of MV release for the host cell, it is now clear that some intracellular pathogens exploit this mechanism for their infection. Notably, CMIRC demonstrated recently that *T. cruzi* metacyclics evade host complement-mediated lysis by fusing with blood cell-derived MVs, which inhibit complement activation by accelerating decay of the C3 convertase[228]. In another study, CMIRC have shown that interaction between *T. cruzi* metacyclics and host cell receptors results in MV production, that causes damage to the integrity of the host cell plasma membrane which the parasite exploits for invasion[172].

Recently, CVBs were reported to specifically exploit Ca^{2+}-mediated signalling events in order to facilitate their entry into polarized endothelial cells[231]. Induction of apoptosis has also been reported as an important step during the infection process, and was shown to elicit an increase of $[Ca^{2+}]_i$ prior to virus entry. An increase in $[Ca^{2+}]_i$ also results in calpain-mediated depolymerization of the host actin cytoskeleton and release of MVs[23].

I first showed that dexamethasone and cyclohexamide can induce release of apoptotic MVs, which carry the death molecules, Fas and caspase-3 that can be transferred to induce apoptosis in healthy cells (**Fig.3.1-3.3**). Moreover, CVB1 (and likely other viral pathogens) can stimulate vaMVs and subsequently exploit host release of the vesicles to enhance viral

transmission and further induction of apoptosis. Using a mouse anti-CVB1 monoclonal antibody by Western blotting and flow cytometry analysis, I showed that these vaMVs carry CVB1 virions that can induce apoptosis in target cells (**Fig.3.4b-e**). In agreement with earlier work [97], vaMVs released by CVB1-infected HeLa cells also carried death signals such as caspase-3 (**Fig3.4e**) that can be delivered to healthy viable cells rendering them apoptotic. I found that addition of the pan-caspase inhibitor, ZVKD-fmk (preincubated at 37°C, 5% CO_2 to encourage endocytosis) reduced entry of CVB1 and subsequent induction of apoptosis in HeLa cells. These vaMVs can also induce production of more MVs, which is abrogated upon inhibition of calpain activation with calpeptin.

In addition, the inhibition of calpain activity by knockdown of *CAPNS1*, severely reduced CVB1 induction of MV release and apoptosis. Moreover, flow cytometry and immunofluorescence analysis confirmed significant reduction in levels of intracellular viruses. Thus this data implies that in addition to utilizing vaMVs for viral spread to new cells, CVB1 also requires host MV production for its initial infection.

Studies with retroviruses have shown that the physical interaction between infected and uninfected cells provide a more efficient way (by 2 to 3 orders of magnitude) for the virus to reach new targets compared to the lytic spread of infection[160, 233]. Moreover, human immunodeficiency virus type 1(HIV-1) exploits depolymerization of the host actin cytoskeleton for intracellular

spread between T cells[234]; thus suggesting a possible role for vaMVs in HIV infection. Viral dissemination via the MV-to-cell mechanism may therefore offer a more unique advantage to the virus, as opposed to transmission using the cell-to-cell strategy. Like viruses inside cells, viral progeny inside vaMVs would also be protected against host-mediated immune responses. Also due to their size (0.1-1 μm diameter), vaMVs can travel to distant regions of the extracellular matrix in order to participate in intercellular communication, which is their principal function. Thus, vaMVs can bring viruses closer to several cell types aiding their infection.

These results offer an explanation to the rapid dissemination of virus particles to secondary sites of infection, and the enhanced induction of apoptosis observed in neighbouring cells during CVB1 infection. I propose a nonlytic MV-to-cell viral spread mechanism by which CVB1 (and likely other viral pathogens) efficiently reach new host cells in contrast to the lytic spread of infection.

4. Cancer cells expulsion of anticancer drugs through shedding of microvesicles: Association with drug resistance and tumour survival

4.1 Introduction

Various findings have reported the occurrence of drug resistance in tumour cells. Drug resistance can be defined as the ability of cancer cells to survive exposure to a wide range of anticancer drugs[180]. Cancer therapy has been abrogated by inherent or acquired drug resistance. Inherent drug resistance results in little or no sensitivity to anticancer drugs in tumours without prior exposure to the drug. Acquired drug resistance is when tumours initially sensitive to chemotherapy later become unresponsive to the drug[131].

Folates, members of the B9 vitamin family, are required in purines, pyrimidines, serines and methionine synthesis which regulate *de novo* synthesis of DNA in mammalian cells[203]. Tumour cells are resistant to drugs such as 5-FU and MTX. Antifolate drugs such as MTX and 5-FU use the same transport system as folates, inhibiting enzymes such as dehydrofolate redoctase (DHFR) and halting folate metabolism. Cells are unable to either synthesize folate or receive these anions through the Reduced Folate Carrier system (RFC)[179].

Drug resistance occurs due to changes in the rate of drug uptake and efflux, altered drug metabolism, decreased drug-target complex formation or enhanced DNA repair mechanisms[190]. Trans-membrane transport proteins and diffusion are two mechanisms involved in anticancer drug entry. For instance, decreased expression of reduced folate carrier (RFC) is associated with MTX resistance[192]. Resistance to docetoxel results from changes in the

protein target caused by mutation at the drug binding site of tubulin and different expression of tubulin isoforms[181]. Multi drug resistance (MDR) develops when tumour cells are resistant to a wide range of chemotherapy drugs. Failure to overcome resistance to chemotherapy is related to the enormous heterogeneity and complex biology of cancer cells and brings about the development of new drugs. However, the new drugs also suffer from high rates of failure and so far have not efficiently reduced the occurrence of drug resistance[189].

The aim of chemotherapy is to induce growth arrest and apoptosis in malignant cells, but the loss of apoptotic function leads to tumour cell survival and resistance to chemotherapy. Several complex pathways and molecules contribute to drug resistance. Mutation in the tumour-suppressor gene p53, is found in more than 50% of cancer types which culminates in multiple recurrences of cancers and development of resistance to the drug[185].

Drug resistance may also be associated with mutation or alteration in the target of anticancer drugs for example resistance to Paclitaxel due to a mutation in the β-tubulin gene leads to inhibition of cell cycle arrest[235]. Each tumour type is different at the histological and molecular levels which makes it even more difficult to find a common mechanism of drug resistance among all cancers[189]. Moreover, the coexistence of multiple defects within a cancer cell contributes to the difficulty of development of effective therapies.

However, advanced technologies will aid identification of new mechanisms and targets that contribute to drug resistance[131].

Microvesicles are intact vesicles shed from cells in health and disease; they vary in size from 0.1-≤1.0 μm. MVs carry various proteins and lipids similar to their cell of origin and are recognized as important mediators of intercellular communication[4]. MVs are released from all cell types and involved in pathological states such as cardiovascular diseases, inflammation, infection, immune disorders and cancers[10]. MVs have a significant role in the tumour microenvironment, progression and metastasis[18]. Cancer cells release MVs to protect themselves against intracellular stress and inhibition of this release leads to accumulation of caspase 3 and cell apoptosis[6].

MVs released from cancer cells containing chemotherapeutic agents, aid drug resistance and interact with the immune system to suppress immune response to tumours[177]. Cancer cells may hide from the immune system by fusing with healthy MVs and expressing their membrane-specific proteins[167]. MVs are also involved in cancer progression through facilitating extracellular matrix degradation[18, 70]

Several factors might contribute to development of antifolate resistance such as increased expression or mutation of cellular targets, intracellular accumulation of tetra hydrofolate cofactors and finally, impaired cellular influx or increased efflux[189, 236]. Here I propose a new mechanism for multi

drug resistance involving the efflux of anticancer drugs from cancer cells mediated by the release of microvesicles and subsequently removal of the drug from treated cancer cells. This mechanism contributes to an understanding of the reasons for insensitivity to drug-induced apoptosis and induction of drug-detoxification by cancer cells. This study has yielded important information about how to circumvent drug resistance to improve cancer chemotherapy.

4.2 Results

4.2.1 THP-1 cells undergo apoptosis after treatment with 5-Fluorouracil.

5-FU is an anticancer drug which is used for the treatment of various types of cancers including colon, rectum, and head and neck cancers. It is classified as an antimetabolite drug. It is cell-cycle specific and halts cell division by acting as a pyrimidine antagonist[131]. To investigate the effect of 5-FU an anti-metaboilite drug on Leukemic cells, first, THP-1 cells (human acute monocytic Leukaemia cells) were selected for the first experiments. In order to ascertain whether 5-FU induces apoptosis in THP-1 cells and whether washing the drug post treatment of the cells, aids their survival, this experiment was performed. THP-1 cells ($5x10^4$ /well) were seeded into 12-well plates in triplicate and were treated with various concentrations of 5-FU. THP-1 cells were washed 30 minutes after treatment with 5-FU and MTX and related condition medium collected to isolate MVs. Cells were washed and resuspended in complete growth medium (GM) and incubated at 37°C with

5% CO_2. Cell viability was determined by Guava flow cytometer with viacount assay every day for 72 h.

Results from Figure **4.1** show that washing THP-1 cells after treatment with 5-FU thereby removing drug-bearing MVs from cells aids cells to survive better and that there is significant difference between cell viability of washed and unwashed THP-1 cells over three days. Cell viability was around 70-80% on the first two days (**Fig.4.1A** and **B**) but there was a significant reduction in cell viability on day three (**Fig.4.1C**).

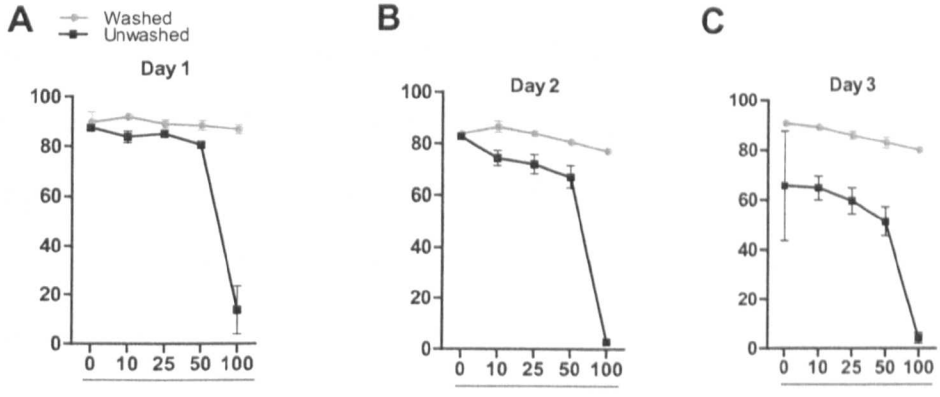

Figure 4.1THP-1 cell viability upon 5-FU treatment. THP-1 cells ($5x10^4$ /well) were treated with different concentrations of 5-FU, in washed conditions, drug was removed after 30 min and cells were washed twice with RPMI and resuspended in GM. Cells were incubated at 37°C with CO_2 for three days and monitored for apoptosis every day. Cells that were washed after treatment with 5-FU survived significantly comparing to unwashed, treated cells, which demonstrates that removing chemotherapeutic drug and MVs released upon drug treatment results in cell survival. Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P = 0.05$ were considered statistically significant.

4.2.2 Calpeptin inhibits release of microvesicles in THP-1 cells treated with 5-Fluorouracil. Observing the results in figure 5.1, I wondered whether cells released MVs upon treatment with 5-FU and whether washing both drug and MVs led to an inhibition of apoptosis. THP-1 cells ($5x10^4$ /well) were seeded into 12-well plates in triplicate. I had washed and unwashed conditions similar to previous experiments but this time cells either were pretreated with calpeptin (CP) to inhibit MV release or left untreated. MV release was inhibited in THP-1 cells pre-treated with CP (20 μM for 45 minutes) prior to induction with various concentrations of 5-FU. Where cells needed to be washed they were then resuspended in GM. The rest of the cells were left unwashed. These results demonstrate that cell viability remained significantly higher in washed cells over three days which further confirms that drug-bearing MVs remove intracellular drug thereby maintaining high cell viability. By contrast, unwashed cells demonstrate increased apoptosis (**Fig.4.2**).

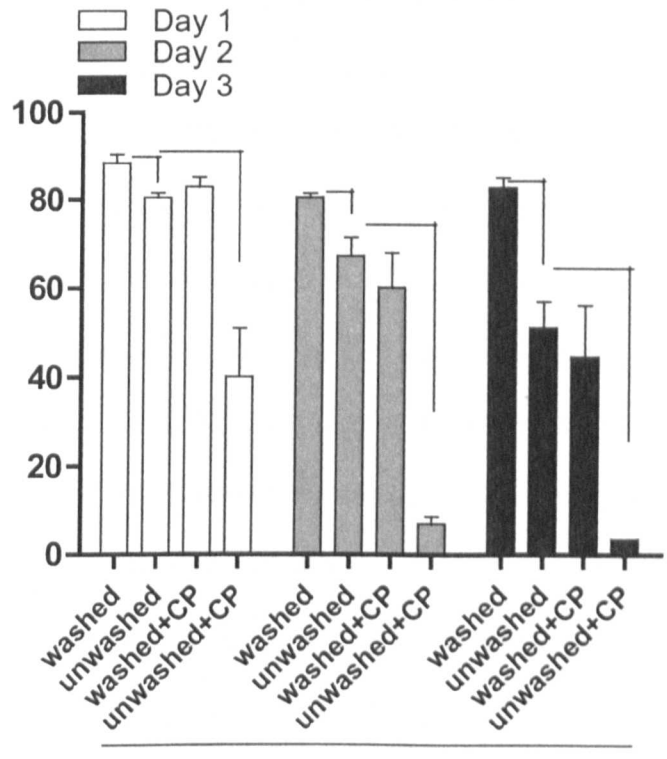

Figure 4.2 Inhibition of microvesicle release in THP-1 cells treated with 5-FU results in cell death. The release of MVs was inhibited in semiconfluent THP-1 cells after preincubation (45 min) with calpeptin (CP)(20 μM). Cells were treated with 5-FU (50 μM) for 30 min and cells either were washed with RPMI twice and resuspended in GM or left unwashed. Cells were incubated at 37°C with 5% CO_2. Cell viability was analysed by Guava viacount assay every 24 h over three days. Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P = 0.05$ were considered statistically significant.

4.2.3 5-Fluorouracil –induced apoptotic MVs deliver death molecules to healthy target cells. To determine whether MVs could transfer the death molecules to healthy cells and thus render them apoptotic, 5-FU- induced MVs were isolated from THP-1 cells. THP-1 cells ($5x10^4$/well) were co-cultured with the apoptotic MVs isolated from conditioned medium of either unstimulated THP-1 cells or cells induced with various concentrations of 5-FU. The experiment was incubated at 37°C with 5% CO_2 for 72 h. Apoptosis was assessed using Guava Nexin Reagent by flow cytometry every day over 72 h. Apoptotic MVs co-cultured with cells induced death of THP-1 cells as compared to control healthy vesicles. Interestingly, 5-FU-induced MVs caused greater apoptosis on day two comparing to day one. Nonetheless, significant levels of apoptosis were observed after 72 h co-culture of apoptotic MVs with healthy cells and this reached approximately 70% (**Fig.4.3**).

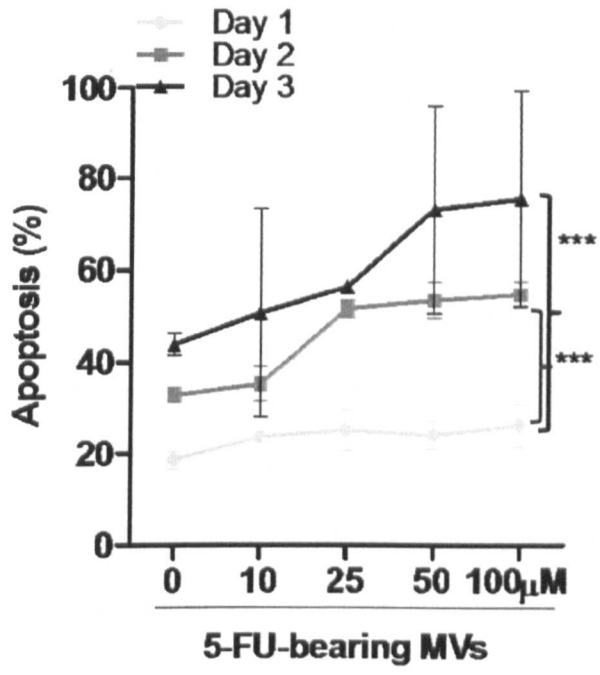

Figure 4.3 5-FU-elicited MVs transfer death molecules to new recipient cells. Semiconfluent THP-1 cells were treated with different concentrations of 5-FU for 30 min then supernatant was collected and MVs were isolated from this supernatant. 5-FU-induced MVs then were co-cultured with healthy THP-1 cells at 37°C, 5% CO_2 for 72 h. Cells were assayed by flow cytometry for apoptosis using Guava Nexin Reagent every day for 72 h. Data presented are mean ± SD of three independent experiments performed in triplicate. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ < 0.001 was considered statistically significant.

4.2.4 Methotraxate induced apoptosis in THP-1 cells. In previous work I have shown that cells treated with 5-FU undergo significant apoptosis when left unwashed. To test whether MTX has the same effect on THP-1 cells I performed the following experiment. In which THP-1 cells ($5x10^4$/well) were treated with various concentrations of MTX. Cells were either washed after 30 min and resuspended in GM or left unwashed. Cells were then incubated at 37°C with CO_2 for 72 h, and then examined for apoptosis using Viacount assay by flow cytometer. Washed cells survived more in comparison to unwashed cells but significant numbers of cells were dead on day three, which indicates the significance of MTX inducing apoptosis compared to 5-FU. There was also a significant decrease in viability on day two compared to day one (**Fig.4.4 A-B**). All cells were dead on day three (**Fig.4.4C**).

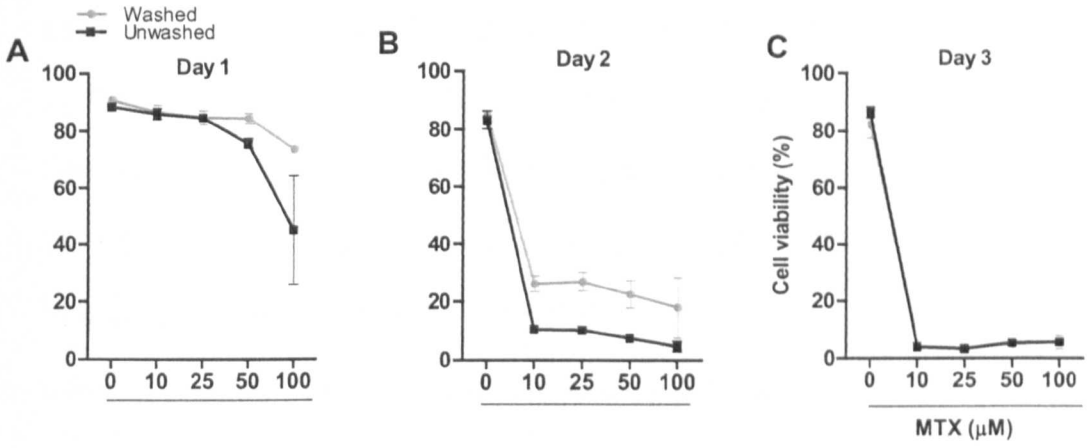

Figure 4.4 THP-1 cells viability after treatment with MTX. The same experiment in figure 4.1 was performed with THP-1 cells ($5x10^4$/well) but cells were treated with different concentrations of MTX. In washed conditions, drug was removed after 30 min and cells were washed twice with RPMI and resuspended in GM. Cells were incubated at 37°C with CO_2 for three days and monitored for apoptosis every day. Treated THP-1 cells were examined for viability using the Viacount assay by flow cytometry every 24 h over three days. Figure **A** shows cell viability was around 80% at the lowest drug concentration and there was inverse correlation between viability and drug concentration. There was a sharp decrease in cell viability on the second day (**B**). All cells were dead on the third day (**C**). Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.5 Inhibition of microvesicle release in MTX treated THP-1 cells using calpeptin. Given the results in figure 4.2 that calpeptin inhibited MV release in THP-1 cells treated with 5-FU, I sought to determine whether calpeptin inhibits MV release when THP-1 cells were treated with MTX. Semiconfluent THP-1 cells were either pr-treated with calpeptin (CP) or left untreated. These cells were then treated with MTX 50 μM. In some conditions cells were washed after 30 min incubation with MTX at 37°C with 5% CO_2. They were resuspended in GM and incubated at 37°C with 5% CO_2 and cells were monitored for apoptosis every 24 h for three days. THP-1 cells were more susceptible to MTX and cell death was significantly increased in unwashed conditions. CP treated cells showed more cell death which indicates that inhibition of MV release leads to an elevated intracellular MTX level, which results in cell death. The majority of cells were dead on day three of experiment (**Fig.4.5**).

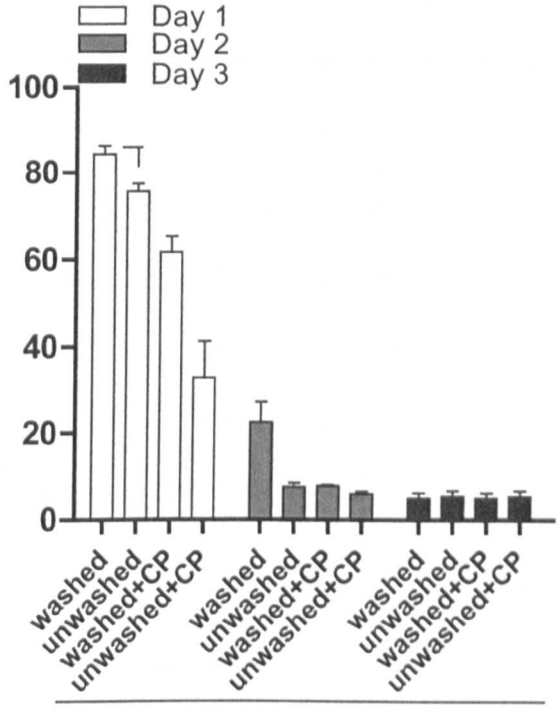

Figure 4.5 Cell viability in THP-1 cells pre-treated with calpeptin and induced with MTX. THP-1 cells ($5x10^4$/well) pre-treated with CP (20 μM for 45 min). Cells were washed with RPMI and resuspended in GM with MTX, 50 μM. Cells were washed twice with RPMI and incubated at 37°C with 5%CO_2 for 72 h. Cell viability was analysed by flow cytometry. Cell viability was significantly higher in washed conditions in comparison with unwashed cells over three days. Apoptosis was significant in cells where calpain inhibition resulted in abrogation of MV release. Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P = 0.05$ were considered statistically significant.

4.2.6 MTX induces release of MVs that carry apoptotic signals and elicit cell death in healthy cells. To further ascertain if MTX-induced MVs transfer apoptotic signals to healthy cells the following experiment was performed. THP-1 cells ($5x10^4$/well) were co-cultured with the apoptotic MVs isolated from conditioned medium of either unstimulated THP-1 cells or cells induced with various concentrations of MTX. The experiment was then incubated at 37°C with 5% CO_2 for 72 h. Apoptosis was assayed using Guava Nexin Reagent by flow cytometry every day over 72 h. Apoptotic MVs co-cultured with cells induced death in THP-1 cells as compared to control healthy vesicles, and interestingly, MTX-induced MVs caused greater apoptosis on day two compared to day one.

These results confirmed that MTX bearing MVs similarly transfer the drug and apoptotic factors of parental cells into their healthy target cells and significantly induce apoptosis over 72 h (**Fig.4.6A**). MVs released from THP-1 cells treated with different concentrations of MTX and 5-FU were isolated and quantified. My results demonstrated that cells treated with 5-FU released more MVs than cells treated with MTX (**Fig.4.6B**), which further confirms previous results that when drug treated cells release more MVs, they survive longer due to removal of the drug through MV shedding (**Fig 4.3**).

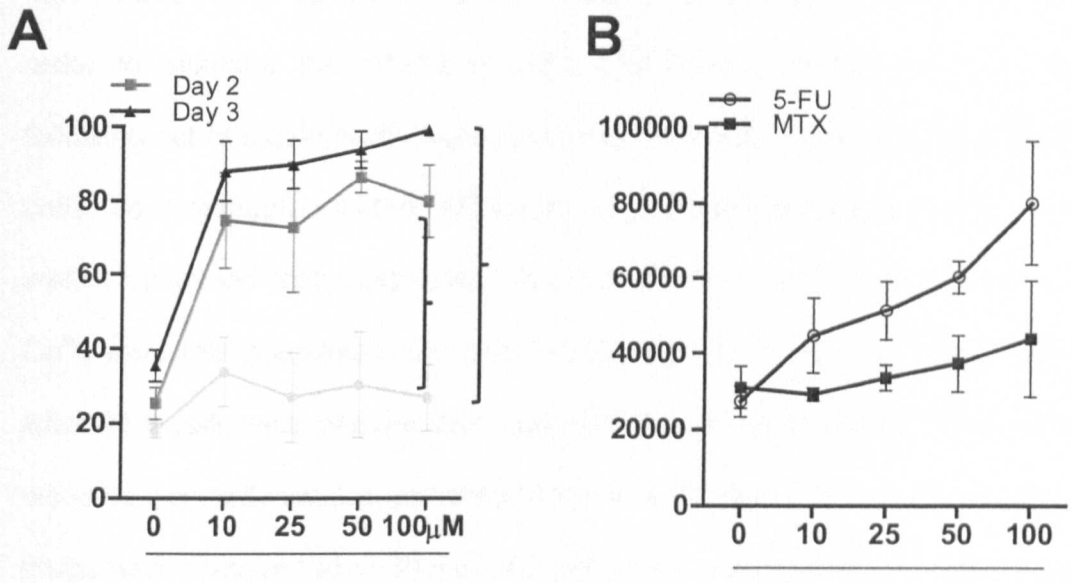

Figure 4.6 MTX and 5-FU induce MV release and THP-1 derived MTX-bearing MVs transfer apoptotic signals. Semiconfluent THP-1 cells were treated with different concentrations of MTX for 30 min, supernatant collected and MVs isolated from the supernatant. MTX-induced MVs were co-cultured with healthy THP-1 cells at 37°C, 5% CO_2 for 72 h. Cells were assayed by flow cytometry for apoptosis using Guava Nexin Reagent every day for 72 h. MVs released from THP-1 cells treated with various concentrations of MTX transfer apoptotic signals to recipient healthy cells (**A**). Flow cytometry analysis of MV production after semiconfluent THP-1 cells were induced with various concentrations of MTX and 5-FU. 5-FU induces more MV release compared to MTX (**B**). Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.7 Induction of apoptosis in PC3M cells treated with MTX and 5-FU. In order to examine the effects of MTX and 5-FU on other cell lines, the following set of experiments were performed on prostate cancer cells (PC3M) cells. To investigate whether MTX and 5-FU induce apoptosis in cells, cells were trypsinised into suspension and seeded into $5x10^4$/well in triplicate. Cells were resuspended in GM and incubated at 37°C with 5% CO_2 for 24 h. After 24 h cells were washed twice with RPMI and resuspended in GM. Cells were treated with various concentrations of MTX and 5-FU. In some plates drugs were removed after 30 min and cells were washed and resuspended in GM. The rest of the plates were left unwashed. Cells were incubated at 37°C with 5% CO_2 for 72 h, flow cytometry analysis then being carried out to measure apoptosis everyday. PC3M cells were more susceptible to MTX than 5-FU. Moreover, washed cells treated with 5-FU showed significant survival rates compared to unwashed cells treated with the same drug (**Fig.4.7.A, B, and C**). Cells washed after treatment with MTX were significantly more viable compared to unwashed cells (**Fig 4.7 D, E, F**).

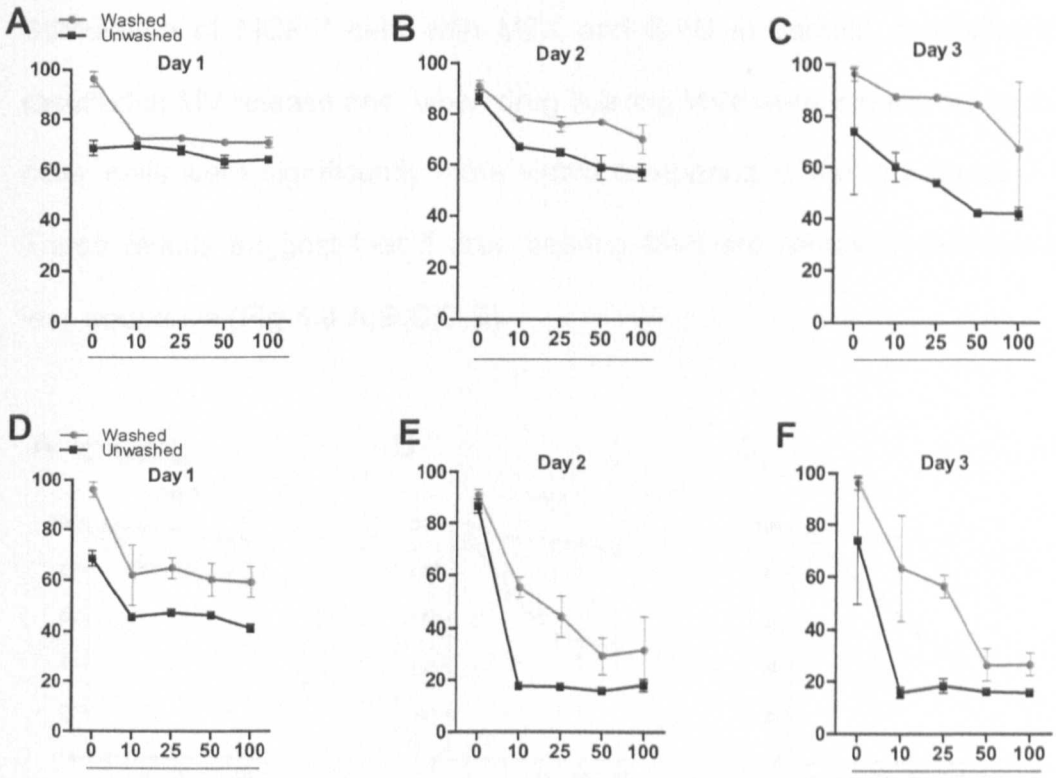

Figure 4.7 PC3M cells treated with 5-FU and MTX undergo apoptosis in a dose-dependent manner. Apoptosis of PC3M cells induced by 5-FU in washed and unwashed conditions was assayed everyday over 72 h using Guava ViaCount by flow cytometry. Cells were significantly viable after washing condition in comparesion to unwashed condition(**A-C**). Flow cytometry analysis of apoptosis in PC3M cells treated with MTX showed significant cell death after washing compared to unwashed condition over three days(**D,E, and F**). Cells were more susceptible to MTX than 5-FU. Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.8 MTX and 5-FU induce apoptosis in MCF-7 cells. MCF-7 cells were trypsinised into suspension and seeded into $5x10^4$/well in triplicate. Stimulation of MCF-7 cells with MTX and 5-FU in various concentrations resulted in MV release and when drug bearing MVs were washed away from cells, cells were significantly more viable comparing to the unwashed cells. These results suggest that if drug bearing MVs are removed cells sustain less apoptosis (**Fig.4.8 A,B,C,D,E**).

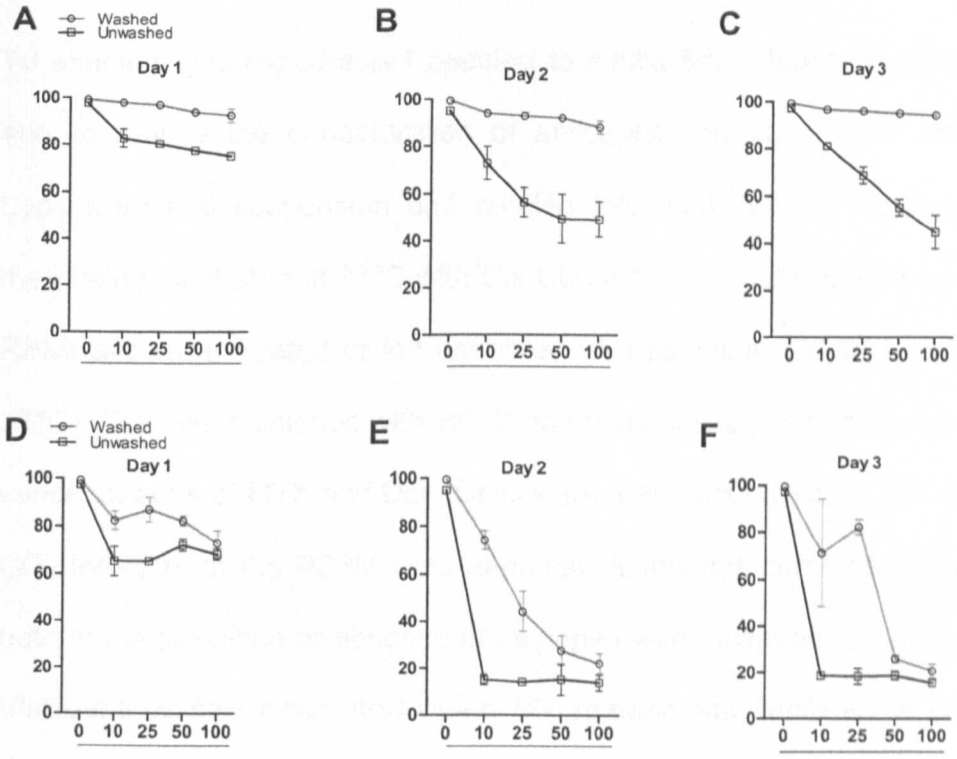

Figure 4.8 MCF-7 cells undergo apoptosis after treatment with 5-FU and MTX. Breats cancer cells also showed more susceptibility to MTX than 5-FU. Apoptosis was assayed by Guava ViaCount by flow cytometry. Cell death was significant in washed cells compared to the unwashed cells. Cells were more susceptible to MTX (**D-F**)than 5-FU(**A-C**) in dose-dependent manner. Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.9 Inhibition of calpain elevates drug-induced apoptosis in PC3M cells. Docetaxel (Doc) is an anticancer drug which is classified as an antimicrotubule agent. Doc arrests cell division by inhibiting the microtubule structures inside the cells[181]. I postulated that MV release in cancer cells upon chemotherapy is a mechanism for expulsion of drug which facilitates cell survival so if MV release is inhibited it might be possible to decrease the concentration of drug needed to achieve significant level of apoptosis.

To examine this hypothesis I decided to inhibit MV release using calpeptin and to reduce the concentration of anticancer drugs. PC3M cells were trypsinised into suspension and seeded into $5x10^4$/well in triplicate, Cells then being incubated at 37°C with 5% CO_2 for 24 h. Cells were washed with RPMI and were treated or left untreated with calpeptin (20μM for 45 min at 37°C). Cells were washed with RPMI and resuspended in GM containing low concentrations of MTX and Doc. Cells were then incubated at 37°C with 5% CO_2 for 72 h. In the PC3M cells apoptosis levels induced by MTX and Doc both in the presence or absence of calpeptin were assayed using the Guava ViaCount by flow cytometry. When MV release was inhibited in cells pre-treated with CP, thereby allowing intracellular levels of drugs accumulate, significant cell death was observed. This results confirm that lowering drug concentration in order to reduce relative side effects is achievable when MV release is inhibited (**Fig.4.9 A-F**).

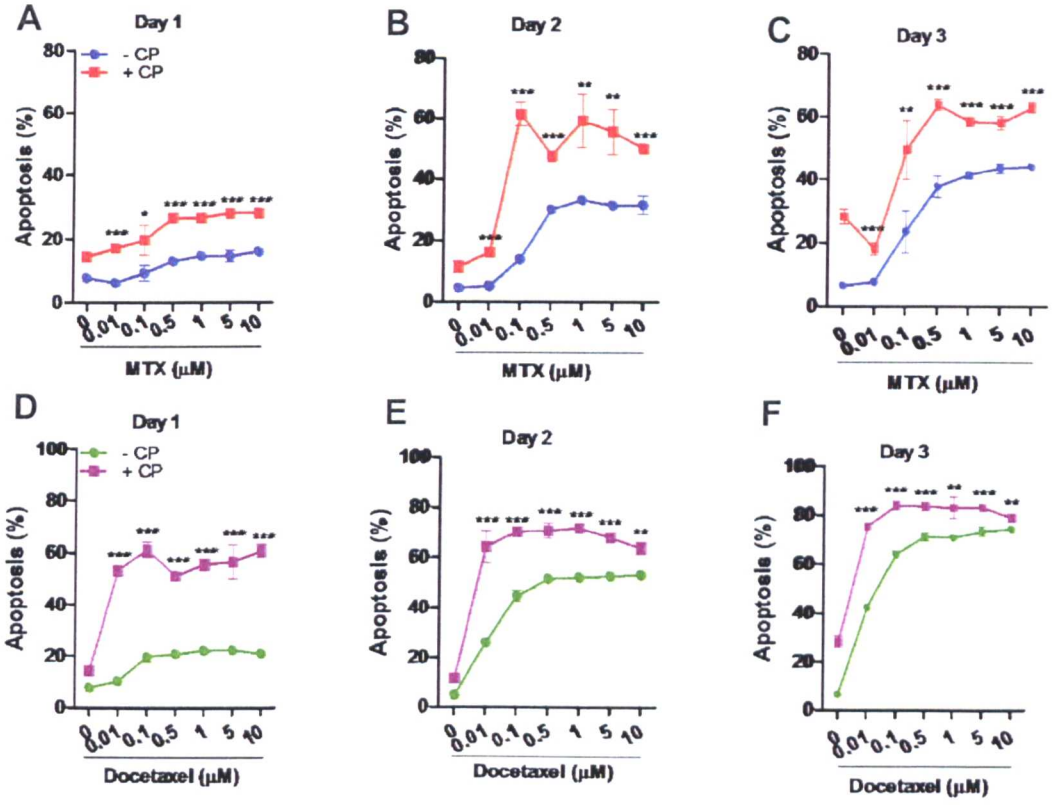

Figure 4.9 MTX and Doc treated PC3M cells undergo significant apoptosis when pre-treated with CP. Semiconfluent PC3M cells were treated with lower concentrations of MTX and Doc in the presence or absence of calpeptin. Cells were assessed for apoptosis with ViaCount by flow cytometry over three days. Pretreatment of HeLa cells with calpeptin significantly abrogated MTX-induced apoptosis (**A-C**). Moreover, Inhibition of MV release in cells treated with Doc showed the same results (**D-F**). Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P = 0.05$ were considered statistically significant.

4.2.10 Elevated cell death is attributed to combined chemotherapy in PC3M cells. Combination of chemotherapy improves survival in patients who suffer from cancer. To investigate whether combined chemotherapy in addition to inhibition of MV release in PC3M cells could reduce the risk of drug resistance, PC3M cells were trypsinised into suspension and $5x10^4$ /well seeded in 12-well plates in triplicate and incubated at 37°C with 5% CO_2 for 24 h. After this period of time cells were washed twice with RPMI and resuspended in GM. Cells were either pre-treated with CP or left without treatment and incubated at 37°C with 5% CO_2 for 45 minutes. Cells were washed and resuspended in GM containing various concentrations of drugs singularly or in combination. Cells incubated at 37°C with 5% CO_2 were then analysed for apoptosis every 24 h for three days.

The results showed that cells treated with MTX+Doc were more apoptotic than cells treated with MTX or Doc individually on day one (**Fig.4.10A**). Cells treated with Doc were more susceptible to apoptosis compared to cells treated with MTX alone or drugs in combination (**Fig.4.10 B, C**). In addition, cells treated with CP+MTX+Doc were significantly apoptotic compared to cells treated with MTX+Doc over 72 h (**Fig.4.10D, E, F**) thus further confirming the role of microvesiculation in expulsion of drug in treated cancer cells.

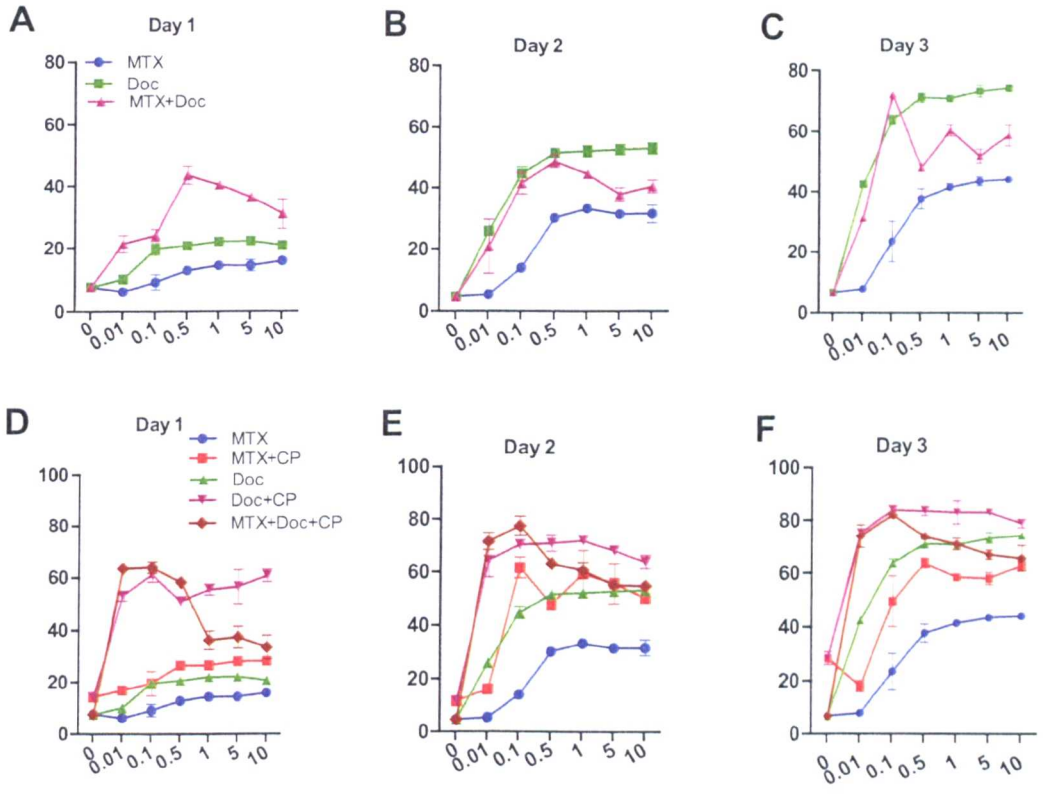

Figure 4.10 PC3M cells treated with Doc and MTX separately or in combination. Cells treated with multidrugs demonstrated significantly more apoptosis than cells treated with single drug. Moreover, pre-treatment of cells with CP prior to treatment with multidrugs increased apoptosis. These results showed that cells treated with MTX+Doc were more apoptotic than cells treated with MTX or Doc individually on day one (**A**). Cells treated with Doc were significantly apoptotic compared to cells treated with MTX alone or drugs in combination (**B, C, D**). In addition, cells treated with CP+MTX+Doc were significantly apoptotic compared to cells treated with MTX+Doc over 72 h (**E and F**). Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.11 MTX-induced MVs, deliver the drug to healthy target PC3M cells.

To further investigate and confirm whether MVs released from cells upon treatment with MTX carry the drug it was decided to show any drug carried by MVs was transferred to recipient cells. To this aim MTX Alexafluor 488 (Lab MTX) was used. PC3M cells ($5x10^4$ /well) were seeded into 12-well plates and incubated at 37°C with 5% CO_2 for 24 h. Cells were washed with RPMI and resuspended in GM with Lab MTX (10 μM) for 60 min at 37°C with 5% CO_2. The supernatant from treated cells was collected and MVs were isolated. These MVs were examined under the fluorescent microscope to observe relative MTX Alexafluor fluorescence (**Fig.4.11B**).The same MVs were analysed by flow cytometer and found to express significant (96%) relative fluorescence compared to healthy MVs isolated from control cells (**Fig.4.11A**). PC3M cells ($5x10^5$/well in triplicate) were co-cultured with healthy MVs, 10:1 (hMVs to cell) ratio or MTX-bearing MVs isolated from PC3M cells treated with lab MTX in a dose-dependent manner. Lab MVs were added to healthy recipient cells in 1:1, 5:1 and 10:1 ratio. These cells were analysed for apoptosis by flow cytometer with ViaCount assay after 48 h. Cells significantly expressed the relative fluorescence in a dose-dependent manner (**Fig.4.11C, D**). **Figure 4.11E** demonstrates significance of apoptosis in recipient cells. THP-1 cells were treated with Lab MTX (0.1 and 10 μM). Released MVs were analysed by flow cytometer and expressed the relative fluorescence 488 (**Fig.4.11F**). THP-1 drug-bearing MVs were added in a 5:1 ratio to healthy THP-1 cells. The flow cytometer analysis of the cells after 48 h revealed significant apoptosis compared to control cells (**Fig.4.11G**).

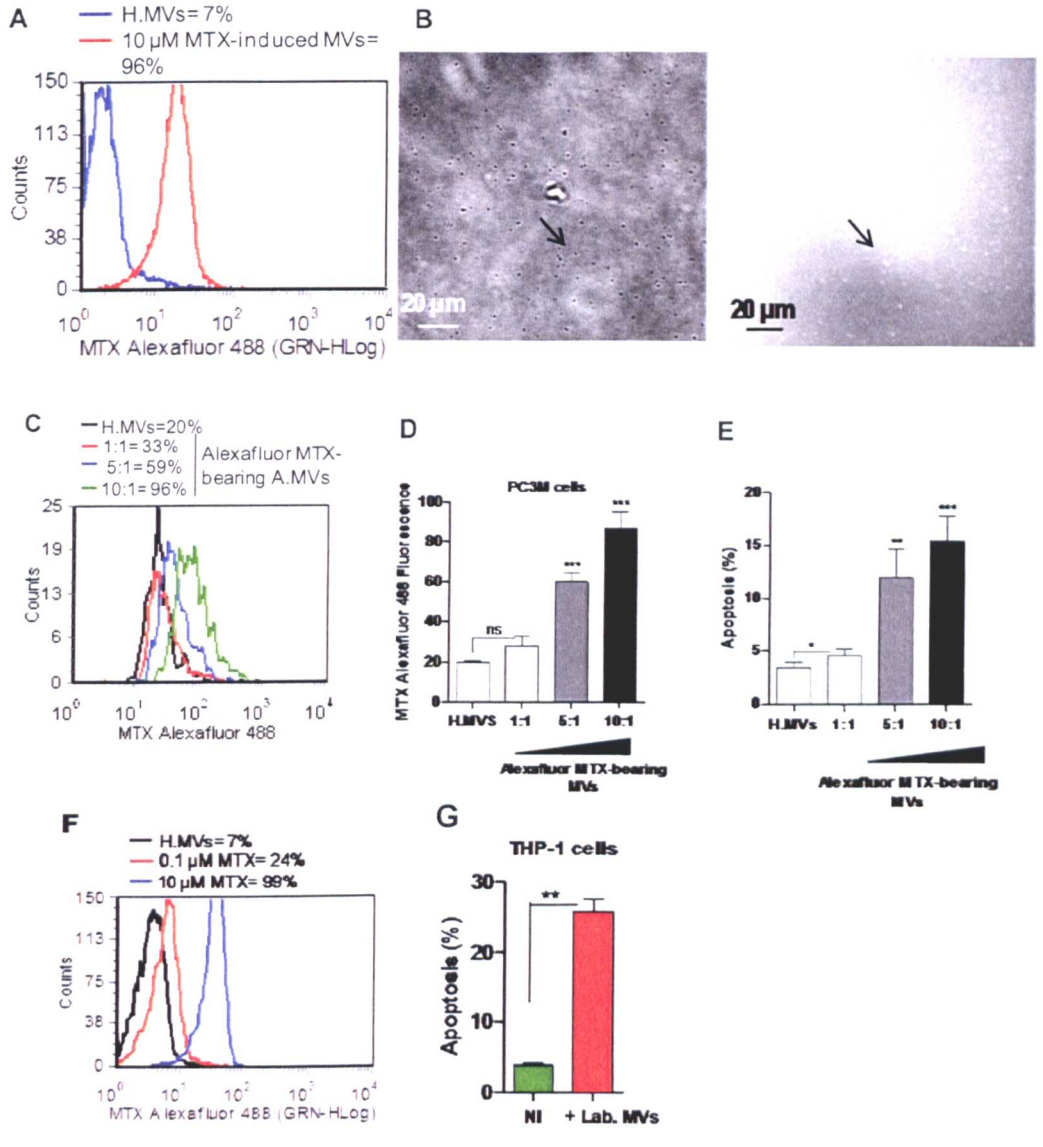

Figure 4.11 PC3M cells and THP-1 cells treated with labelled MTX express fluorescently labelled MTX and MVs isolated from these cells carry fluorescent drug and transfer it to recipient cells. A labelled MTX Alexafluor 488 was used to treat PC3M and THP-1 cells. MVs from these cells were isolated and added to recipient healthy cells in different ratio (**A, B, C, F**). Both cells and related MVs expressed relative Alexafluor fluorescence 488 which demonstrates that MTX bearing MVs are released upon cell induction with labelled MTX and transfer labelled drug and apoptotic signals into recipient healthy cells (**D, E, G**). ***P < 0.001; **P < 0.01; *P < 0.05 were considered statistically significant.

4.2.12 Downregulation of calpain expression inhibits drug-induced apoptosis in PC3M cells. To investigate the role of calpain in MV release, a knockdown of both μ- and m-calpain isoforms using calpain small-subunit 1small interfering RNA (*CAPNSI siRNA*) was carried out. PC3M cells were transfected with siRNA *CAPNS1* and incubated at 37°C with 5% CO_2 for 48 h. Cells were fixed and permeabilised. Cells were labelled with anti-CAPNS1 antibody and analysed for relative *CAPNS1* expression using Express plus assay by flow cytometry to determine effectiveness of silencing. Notably, knockdown of *CAPNS1* which leads to abrogation of MV production resulted in a dramatic reduction of *CAPNS1* expression (**Fig. 4.12A and B**).

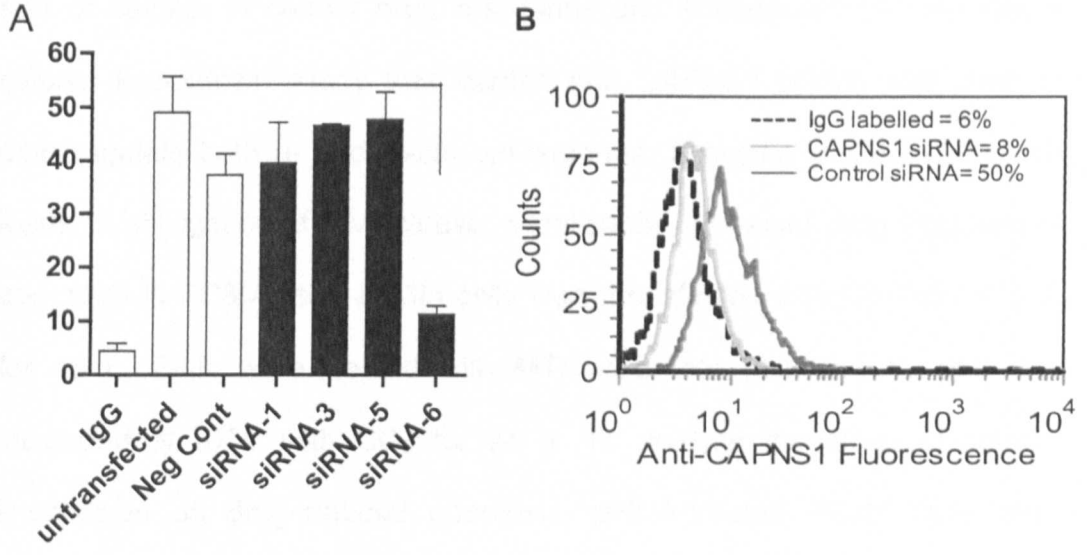

Figure 4.12 Calpain silencing in PC3M reduces *CAPNS1* expression. Semi-confluent PC3M cells were transfected or not with 50nM of control (Neg Cont) siRNA or various *CAPNS1* siRNA sequences targeting different regions for 48 h. Flow cytometry analysis of *CAPNS1* knockdown in siRNA treated cells showed that siRNA-6 had the most significant reduction of *CAPNS1* expression. To further confirm that siRNA-6 is the best choice for silencing calpain, PC3M cells were transfected with siRNA-6 (50nM) for 48 h. Relative *CAPNS1* expression assessed by flow cytometry showed a 42% of reduction compared to negative controlled siRNA(**A,B**). Data presented is the mean ± SD of three separate experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.13 Effect of calpain silencing on drug resistance. To investigate the role of calpain in cancer drug resistance and subsequent MV release, a calpain knockdown assay was carried out. *CAPNS1 siRNA* was used to downregulate both μ- and m-calpain isoforms. Silencing of *CAPNS1* which leads to abrogation of MV release, significantly increased drug induction of apoptosis in PC3M cells. PC3M cells were transfected with *CAPNS1 siRNA* for 48 h. Cells were treated with MTX and Doc (0.1 and 10 μM) and incubated at 37°C with CO_2 for 24 h. To analyse the effect of calpain knockdown on drug-induced apoptosis, siRNA-treated PC3M cells were treated with various concentrations of MTX and Doc and were assessed by Guava ViaCount assay and analysed by flow cytometry. Transfected cells were more susceptible to the drugs than control cells. This emphasises the importance of MV release in cancer cell survival. Inhibition of MV release in PC3M cells lead to increased cell death due to an inhibition of the ability of cells to export the drugs by microvesiculation. This mechanism aids comprehension of drug resistance in cancer cells (**Fig.4.13A-D**).

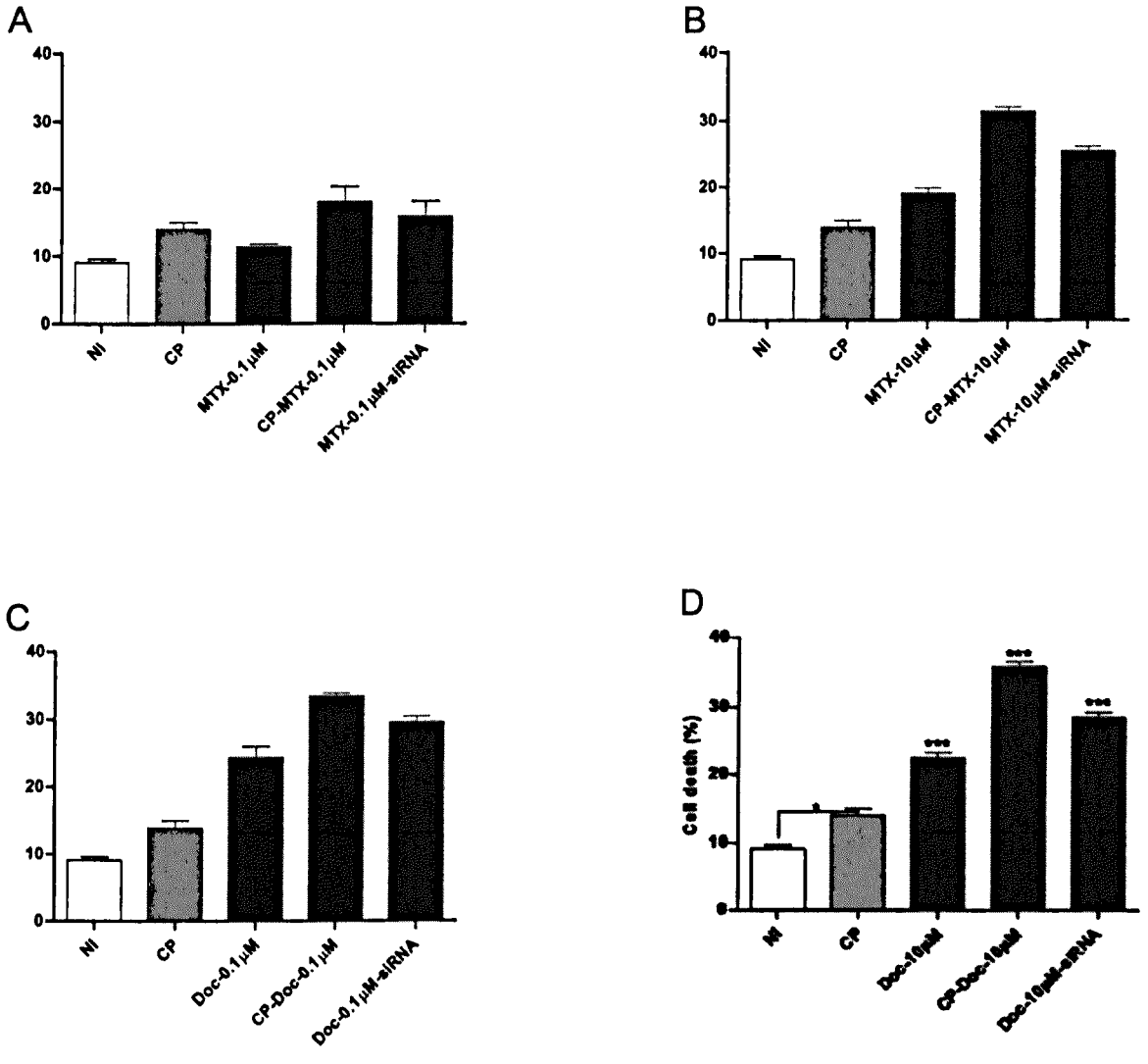

Figure 4.13 Silencing Calpain in PC3M cells results in elevated levels of cell death. PC3M cells were transfected with *CAPNS1* siRNA-6 (50nM) for 48h. Cells were washed with RPMI and resuspended in GM with either MTX (0.1 and 10 μM) or Doc (0.1 and 10 μM) and incubated at 37°C with 5%CO_2 for 24h. Cells were analysed for apoptosis using the Viacount assay by flow cytometry. Silencing calpain in PC3M cells significantly led to a noticeable increase in drug induced apoptosis in cells treated with MTX (**A-B**) and Doc (**C-D**). Data presented are mean ± SD of three independent experiments performed in triplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically significant.

4.2.14 Inhibition of MV release significantly decreases drug concentration needed in an *in vivo* model of prostate cancer. In a xenograft model of human prostate cancer, male athymic nude mice were injected subcutaneously with $5x10^6$ PC3M cells, once the tumour volumes attained an average of 100 mm^3, once weekly therapy was initiated as seen in **Fig.4.14**, treatment with 10 mg/kg calpeptin enabled a 10-fold lower dose of both docetaxel (Doc) (**A**) and methotrexate (MTX) (**B**) to produce significant reductions in tumour volume. Plasma MV levels showed a concomitant decrease upon injection of calpeptin (**C**). Excised tumours showed significant reductions in size and weight (% tumour weight inhibition) upon treatment with Doc or MTX in combination with calpeptin (**Fig. 4.14 A, B and D**). Markers of cell proliferation, apoptosis and angiogenesis are now being assessed in these tumours.

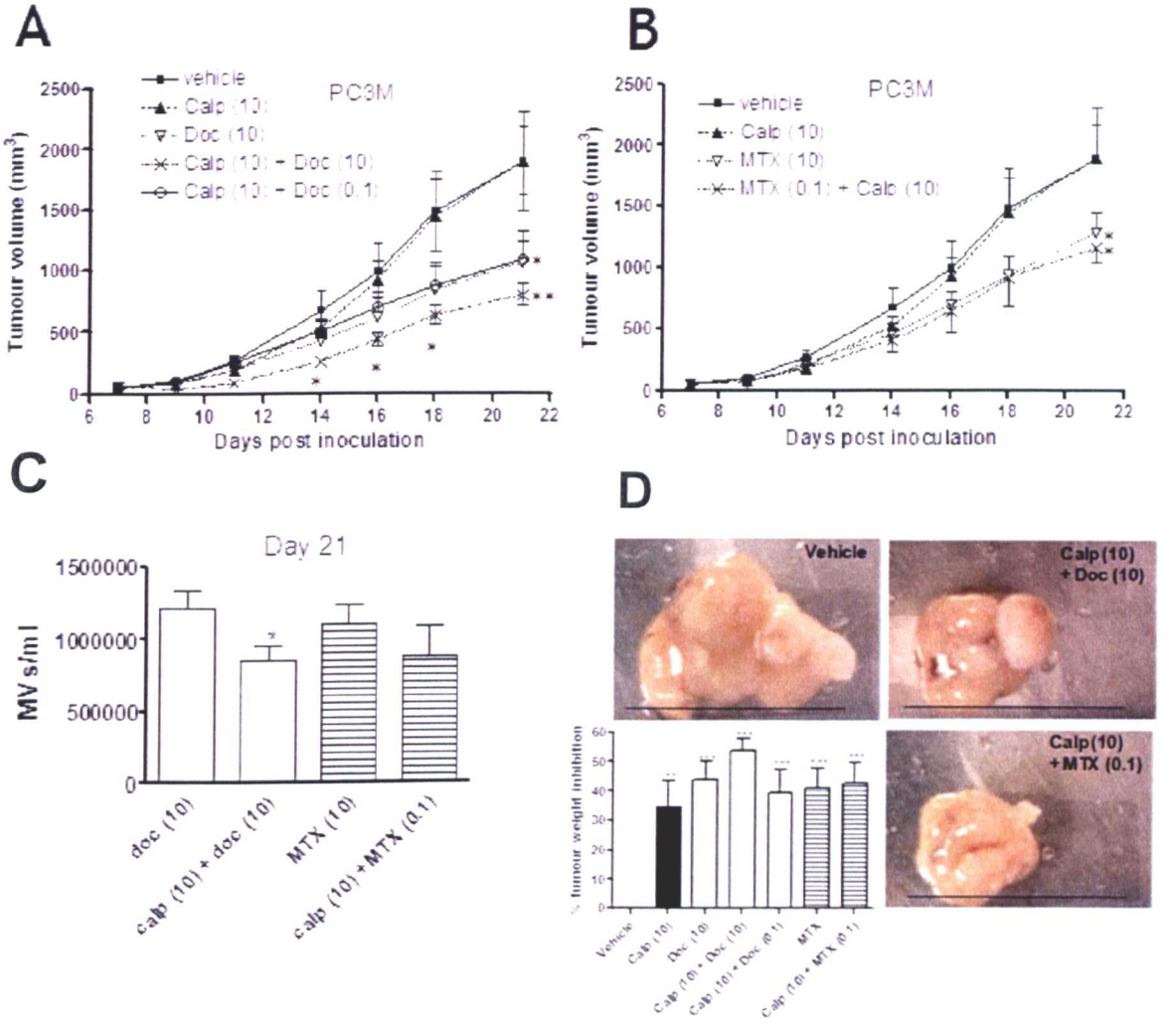

Figure 4.14 Mouse model of human prostate cancer was designed and further confirmed *in vitro* results. Male athymic nude mice were injected with PC3M cells. Once the tumour volumes reached 100mm^3, treatment with 10mg/kg calpeptin and various dosages of MTX and Doc was initiated. Tumours treated with calpeptin and drugs in combination showed significant reduction in volumes compare to control (**A and B**). MVs were quantified in plasma sample of mice on day 21 and mouse treated with calpeptin and Doc (10µg/kg) showed significant reduction of MVs(**C**). Figure **D** demonstrates images of tumours which were reduced in size upon treatment with combination of drugs and calpeptin, percentage of tumour weight inhibition also further confirmed that combination of MTX and Doc with calpeptin significantly inhibits tumour growth.

4.2.15. An *in vivo* mouse model of prostate cancer and the effect of drug toxicity on body weight over 3 weeks. In a xenograft model of human prostate cancer, body weight did not fall during the 3-week course of the experiment (**Fig. 4.15 A and B**) indicating there was no toxicity with the treatment.

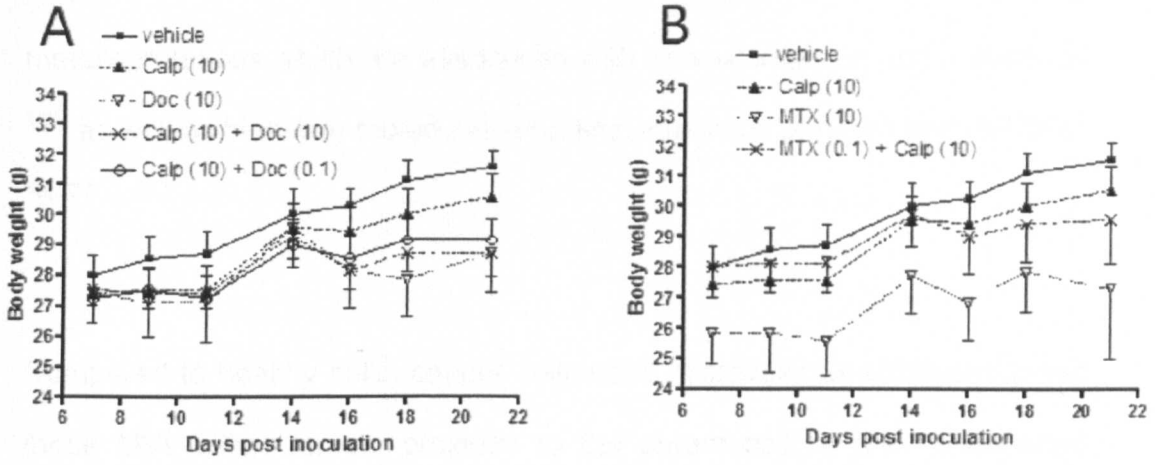

Figure 4.15 Mice body weights over 3 weeks of experiment. The body weight of mice treated with Doc, calpeptin individually or in combination (**A**) and with MTX, clapeptin inidividually or in combination(**B**) were measured over the 3 weeks of experiment and it was found not to fall, suggesting that any reductions in tumour size were not due to toxic effects of the drugs.

4.3 Discussion

Microvesicles are small membrane-coated vesicles present in blood, urine and other body fluids and carry cargo which depends on the cell type they originate from[1]. Their role in diagnosis, prognosis and in surveillance of diseases has heightened their importance[9]. MVs released from cancer cells have been implicated in angiogenesis, metastasis and evasion of immune responses[18, 119, 168]. Tumour cells also shed microvesicles carrying metalloproteases which are associated with tumour invasion and metastasis [119], as well as bioactive molecules, proteins, infectious particles and mRNAs[5, 18, 237].

Compared to healthy cells, cancer cells shed microvesicles excessively and these MVs either remain proximal to the parent cell or are transported through biological fluids[18]. MVs traffic their cargo to new sites and can transmit mRNA, microRNA and even oncogenes to recipient cells[1, 175].

Multi-drug resistance (MDR) has been defined as the ability of cancer cells to survive after treatment with various drugs and present a major obstacle in cancer chemotherapy[189]. However, the mechanisms used by cancer cells to evade apoptosis induced by anticancer drugs remain unclear and was the subject of this investigation. The active efflux of broad range of anticancer drugs via the cell membrane is aided by multi drug resistance proteins via ATP-dependent and independent manners in tumour cells[238]. These proteins are members of the ATP–binding cassette transporter family including P-gp,

MRP, BCRP[191, 193, 238]. Lung resistance-related protein (LRP) however is another protein involved in multi-drug resistance not belonging to the ABC transporter family. These proteins are involved in uptake and efflux of substances from cancer cells[182].

Microvesicle trafficking is a significant process in tumourigenesis so its alteration can contribute to multidrug resistance[70]. MVs carry and transfer adhesion molecules to target cells, which provide a platform for target cell binding[70]. For the first time Bebawy M et al reported on 'non-genetic' mechanism for MDR where MVs transferred P-gp from MDR leukemic cells to drug-sensitive target cells[132]. The involvement of both P-gp and MRP1, as efflux transporters have been reported in MDR[193].

These findings were considered in the present study and led to new hypothesis to examine whether MVs released upon cancer cells treated with chemotherapeutic agents carry drugs and transfer them to recipient cells and if inhibition of MV release could be considered as a potential alternative treatment method in tackling MDR. Calpain is a Ca^{2+}-dependent cysteine protease, once it is activated, it cleaves cytoskeletal actin filaments leading to release of MVs[43]. The involvement of calpain in MV release has been demonstrated in previous studies where in thrombotic thrombocytopenic purpura (TTP) the presence of circulating calpain activity in the plasma was strongly associated with the release of platelet MVs[43]. Eukaryotic cells require both intracellular transport and exportation of substances to

extracellular matrix. It has been demonstrated that shedding MVs deliver their components upon fusion with an acceptor target membrane[6]. MTX and 5-FU, antimetabolites drugs have been implicated in MDR[131]. MTX resistance has been described as result of decreased expression or complete inactivation of the RFC receptor suggesting that diminished uptake by RFC develops a significant general resistance to antifolates[203]. An irreversible inhibitor of thymidylate synthase 5-fluorouracil (5-FU) is anti-metabolite drug which is degraded in liver by dihydropyrimidine dehydrogenase (DPD). Resistance to 5-FU has been described as a result of over expression of DPD in cancer cells[179].

The cell cytoskeleton is associated with cell signalling, cell division, mitosis, cell shape, and transportation of vesicles and mitochondria. Polymerisation of microtubules is essential for successful spindle function and chromosome segregation in mitosis[239]. Docetaxel is member of taxanes family which inhibits microtubule polymerization and halts mitosis at the metaphase-anaphase leading to apoptosis. Changes in microtubule polymer mass and expression of various microtubules are associated with development of antimicrotubule resistance[181]. Although these findings have contributed greatly to our understanding of MDR, the empirical data presented in this study demonstrates a novel mechanism and association of MV release with drug resistance.

Furthermore, the treatment of cancer patients with methotrexate or docetoxel leads to significant side effects due to the use of higher doses. Here I show that these drugs when administered synergistically with calpeptin could be given at doses 100 times less and still induce effective killing of target cancer cells. This suggests a number of strategies that may be important for cancer therapeutics. Overall I report a novel mechanism involving cancer cell expulsion of anticancer drugs via the release of MVs, followed by the recruitment of lysosomes to the site of release to repair the resulting damage. In addition, I show for the first time that inhibition of MV release by pretreatment of PC3M cells with the calpain inhibitor, calpeptin or by silencing calpain gene sensitizes cancer cells to drug-elicited apoptosis mediated by the addition of methotrexate and docetoxel at lower concentrations.

This study was initiated with experiments using THP-1 cells which were treated with increasing concentrations of 5-FU. Washed and unwashed cells upon treatment were measured for apoptosis every day for 72 h. As previously shown MV release is initiated with significant changes in intra cellular Ca^{2+} homeostasis due to either ER localized Ca^{2+} or mitochondrial Ca^{2+} release, resulting in the activation of the Ca^{2+}-dependent cystein protease,calpain.

Calpeptin, the inhibitor of calpain inhibits microvesiculation[42]. At the outset this study found a significant level of apoptosis in unwashed cells compared to washed cells which indicates that removal of MVs and drug aids cell survival (**Fig.4.1A-C**). The same experiment was performed but this time under certain conditions cells were pretreated with calpeptin to inhibit microvesiculation. Once again unwashed cells pretreated with calpeptin showed an increased level of apoptosis compared to unwashed cells without calpeptin treatment. This emphasises the role of MV release in cancer cells survival (**Fig.4.2**). Previous studies have shown a role for MVs in MV-induced intracellular cross talk[240] and recent studies confirmed that release of MVs is associated with transfer of antigens and receptors to initiate cell signaling[105].

When MVs released from unwashed cells treated with drug alone were isolated and added to recipient healthy THP-1 cells, the recipient cells showed increasing levels of apoptosis over 72 h meaning that these MVs had taken away active drug (which was capable of transferring apoptotic signals to their target cells) (**Fig.4.3**).

The same experiments (washed and unwashed with or without calpeptin) were performed with MTX on THP-1 cells and similar results were obtained, inhibition of MV release resulting in significant cell death (**Fig.4.4A-C and Fig.4.5**); again MVs shed from THP-1 cells treated with MTX transferring apoptotic signals to recipient cells (**Fig.4.6**). To further confirm the results

from previous experiments, the same experiments were performed with similar results on PC3M and MCF-7 cells (**Fig4.7A-F, 4.8A-F**).

It was found that lower concentrations of MTX and Doc significantly increased cell death when microvesiculation was inhibited in PC3M cells over 72 h (**Fig.4.9A-F**). These results confirm that the release of MVs aids efflux of drugs from cancer cells which contributes to drug resistance and when MV shedding is inhibited, drug cytotoxcity is enhanced even though the drug concentration is lower. In agreement with earlier work, cell death was significantly increased (upon inhibition of calpain) in cells treated with a combination of drugs and calpeptin (**Fig.4.10E-F**).

It has been documented that MVs transfer surface receptors to local cells or deliver proteins or mRNA to target cells[58]. To investigate whether MVs can carry an anticancer drug and remove it from treated cells, it was shown that when PC3M and THP-1cells were treated with labelled MTX, the MVs released expressed MTX relative fluorescence (**Fig.4.11A-B**). These MVs bearing labelled MTX transferred the drug to recipient healthy cells in a dose-dependent manner (**Fig.4.11C**). Moreover, cells co-cultured with drug bearing MVs expressed relative MTX fluorescence and showed significant levels of apoptosis (**Fig.4.11D, E, G**).

One mechanism that contributes to cancer drug resistance is cancer cells ability to evade chemotherapeutic treatment by increasing active drug efflux

through MV shedding[132]. Calpain, a Ca^{2+}-dependent protease is activated in cells as a consequence of stimulation of cells by agonists[44]. Cytoskeletal rearrangement due to microvesiculation is inhibited by a membrane-permeant calpeptin[43]. The results generated show that calpain inhibition by calpeptin in drug treated cancer cells induces apoptosis. This time to further investigate the role of calpain in drug resistance, experiments focused on inhibition of calpain activity by silencing *CAPNS1* (**Fig.4.12 A, B**). Transfected PC3M cells treated with drug showed significant cell death, which further confirms the role of drug expulsion through MV blebbing and an enhanced cell survival (**Fig.4.13A-D**).

Experiments were then designed to confirm these findings in a mouse model of human prostate cancer. The data presented showed that tumours treated with calpeptin and therapeutic agents had a significant reduction in size and volume (**Fig.4.14A, B**). MV quantification in mouse treated with calpeptin and Doc showed significant reduction on day 21(**Fig.4.14C**). Tumour sizes were reduced upon treatment with combination of drug and calpeptin and this was further confirmed by a significant inhibition of tumour growth and weight (**Fig.4.14 A,B,D**). Body weights did not decrease over the three weeks of the experiments (**Fig.4.15 A, B**). These studies shed light on the role of MV release in cancer cell expulsion of anticancer drugs and subsequent evasion and survival from apoptosis. An expulsion of therapeutic drugs from tumour cells through shedding of MVs culminates in tumour-cell survival. Cancer cells shedding MVs bearing MTX and Doc act as a drug-efflux mechanism

involved in drug resistance which shows that MVs have an enormous impact on tumour growth and survival.

5. Host cell release of microvesicles, causes damage to the cytoskeleton and leads to activation of Ca^{2+}-regulated lysosomal repair

5.1 Introduction

Microvesicles (MVs) are small (0.1-≤1 μm in diameter), intact, membrane vesicles released constitutively, or upon activation with extracellular stimulus. The process is dependent on an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$), which activates the enzyme, calpain, resulting in depolymerization of the actin cytoskeleton and release of the vesicles[10]. Epithelial cells are constantly exposed to various stresses, *in vivo*, resulting in increased $[Ca^{2+}]_i$ and subsequent release of MVs from the cell surface. Detachment of MVs from the parental cell can be envisaged as plasma membrane (PM) disruptions that require rapid resealing. Repairing these surface disruptions is necessary for survival of wounded cells, as failure results in necrotic cell death. Resealing of membrane disruptions require Ca^{2+}-regulated exocytosis of organelles such as lysosomes to the wound site[208].

In eukaryotic cells survival depends on host cells maintenance of an intact plasma membrane (PM). Disruptions to the PM of eukaryotic cell interfere with its integrity, which if not resealed allows the unabated influx of potential toxins such as high concentrations of Ca^{2+} to flood into the cytoplasm of the damaged cell[215]. Plasma membrane resealing is a Ca^{2+}-dependent mechanism that involves the exocytosis of intracellular vesicles to the site of damage. Calcium regulated exocytosis is not a unique process only present in defective cells, but also a mechanism utilized by normal cells[205].

Depending on their localization within the organism, many cells are likely to experience membrane damage on a frequent and recurring basis. Indeed, it can be considered natural for cells to undergo membrane injury if placed in harm's way. Epithelia, especially those of gastrointestinal tract and lungs are subject to continual mechanical disruption, the endometrial lining of the uterus undergoing cycles of destruction and repair, whilst membrane fissures are the result of mechanical strain in skeletal muscles and immune cells are attacked by toxin secreted by invading pathogens and/or the host's own defence systems such as perforins and blood complement complexes[208, 210].

One membrane repair mechanism involves conventional lysosomes responding to Ca^{2+} by fusing with plasma membrane. By way of support, modulation of synaptotagmin VII function through gene knockout analysis, revealed a role for lysosomal exocytosis in the repair of plasma membrane lesions[241].

Here, I tested the novel hypothesis that the release of MVs, although, essential for intercellular communications, result in damage to the host PM that requires rapid repair.

The involvement of calcium ions in the release of MVs has been well documented in the literature[2, 25]. Stimulation of mammalian cells in the presence of the calcium chelator, EGTA, is known to inhibit release of MVs. An increase in intracellular calcium levels, results in rearrangement of the actin cytoskeleton, followed by release of MVs[44]. Most of the calcium

channels reported to play a role in the rearrangement of the cytoskeleton refers to L-type channels[44]. In addition to L-type calcium channels, there is possible involvement of other channels, such as mechanosensitive channels (MSC) and in particular stretch-activated channels (SAC). Mechanosensitive channels (MSC) are ubiquitous ion channels, which serve many functions in cells and higher organisms[242]. The release of MVs is sometimes activated by the sheer stress sustained by the host cell in the presence of the inducing agent[10], so activation of such channels may be associeted with microvesiculation.

To investigate how MV release results in PM damage it was necessary to uncover how mammalian cells repair plasma membrane breakage. A break in the plasma membrane of a eukaryotic cell interferes with its integrity and immediately compromises the essential role of this structure as a barrier, and this can cause death to the affected cell due to excess influx of substances such as $Ca^{2}+$[208]. A calcium-dependent mechanism was proposed, which involves rapid resealing of the disruption, by exocytosis of activated lysosomes[207]. Influx of calcium through the breakage recruits lysosomes, which fuse together and patch-up the site of damage. This mechanism of wound repair has been reported in skeletal muscle cells, gut, skin and aortic endothelium cells[209]. Moreover, recent findings suggest the same mechanism can be used to explain processes by which cells repair larger surface lesions caused by pore-forming proteins, such as the complement MAC and the bacterial toxin streptomycin O (SLO)[243]. In this study, MV release

significantly enhanced by disruption of host cell microfilaments, due to treatment of cells with anticancer drugs. During treatment of cell with anticancer drugs, host cell lysosomes were shown to gradually accumulate and progressively fuse with the plasma membrane. Subsequent studies demonstrated that fusion of host cell lysosomes with the plasma membrane is activated by elevations in cytosolic Ca^{2}+ levels stimulated by anticancer drugs[205]. Here, I present evidence that the release of MVs from cells induced by NHS or these drugs, causes damage to the integrity of the host plasma membrane before lysosomal repair.

The release of MVs is a process that follows depolymerization of the actin cytoskeleton, which is itself caused by activation of signalling cascades induced by increases in cytosolic Ca^{2+}[23]. Increase of cytosolic Ca^{2+} concentration is in part caused by activation of host cell integrin receptors and lipid raft microdomains by MV release[51]. For this reason experiments were designed to investigate the role of MVs upon treatment of the cells with anticancer drugs. It is important to emphasise that this study is the first to report a possible link between cancer drug resistance and release of MVs. This study also offers a possible explanation as to why it is essential for cancer cells to stimulate the release of MVs post chemotherapy and repair PM. In this thesis, MVs-free NHS was used for cell induction.

5.2 Results

5.2.1 Dot plot distribution of MVs on flow cytometer. Isolated MVs characterised by flow cytometry, showed a typical dot plot distribution. Debris-free conditioned medium sonicated in a sonication water-bath to separate aggregated exosomes, was centrifuged at 25,000 *g* for 2 h to pellet MVs. MVs isolated are depicted simply by their distinct, heterogeneous forward and side scatter distribution by a flow cytometry dot plot (**Fig.5.1A**). This plot shows forward scatter (size) and side scatter (granularity), and the heterogeneity in size of the MVs. Phosphatidylserine expression on the membrane surface indicates the loss of lipid membrane asymmetry, this being the main characteristic of cells undergoing early apoptosis[13] . However, during release of MVs, the loss of cell asymmetry, results in inversion of the two membranes on the vesicles. Phosphatidylcholine (PC) and sphingomyelin carried on the outer leaflet are inverted inwards, while phosphatidylethanolamine (PE) and phosphatidyleserine (PS) in the inner leaflet are exposed on the external surface[176]. Using flow cytometry, isolated MVs from THP-1 cells were found to express PS on the outer leaflet by staining with annexin V-FITC comparing to control (**Fig.5.1B**).

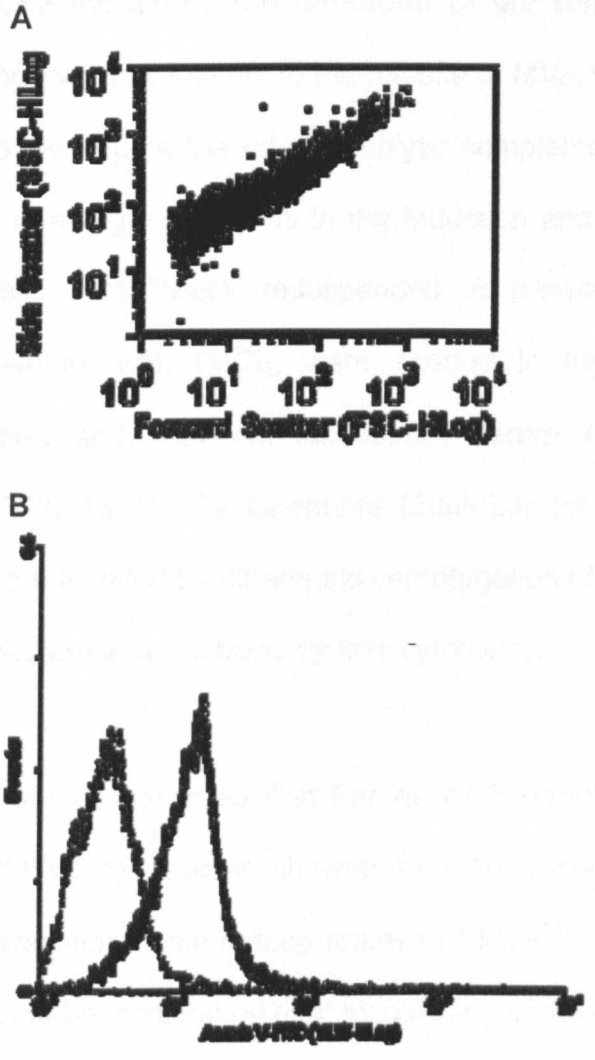

Figure 5.1 MVs may be identified by a classic dot plot distribution and by high expression of phsphatidylserine. (**A**) MVs were isolated by differential centrifugation of THP-1 cell-depleted conditioned medium and analysed by flow cytometry. The vesicles were identified by their size and granularity, as assessed by the logarithmic amplification of forward (FSC) and side scatter (SSC) signals. In (**B**) the THP-1 monocyte-derived MVs show strong positive fluorescent staining with Annexin V-FITC indicating a strong exposition of phosphatidylserine on the outer leaflet of the released MVs.

5.2.2 Induction and inhibition of MV release. To further understand the mechanisms leading to the release of MVs, the next experiments were set up to investigate the role of sublytic complement in form of NHS and agonists and antagonist agents in the induction and inhibition of MV release. THP-1 cells ($1x10^6$/well), resuspended in prewarmed (37°C) serum-free RPMI medium with $CaCl_2$, were seeded in triplicate into 24-well plates and stimulated with normal human serum (NHS; 10%), BzATP (300μM), ATP(300mM), Ca Ionophore (20μM);all for 30min at 37°C. Released MVs, were isolated by differential centrifugation of THP-1 cell-depleted conditioned medium and analysed by flow cytometry.

It should be noted that Far as NHS-stimulated MV release is concerned; CMIRC lab earlier showed that NHS contains sublytic MAC and MAC deposition could induce release of MVs[172]. This is because the MV release could be inhibited by heat inactivating of the complement (56°C for 30 min) or by depleting complement C9. Wells containing cells only (without NHS) were used as negative controls. Different groups have reported the use of EGTA, a calcium chelator in reducing the amount of MVs released by platelets. In this study, EGTA also inhibited release of MVs in the cell-lines tested (**Fig.5.2A**). Cells pretreated with calpeptin at 37°C with CO_2 for 45min, and then were treated with NHS, BzATP and ATP for 30 min at 37°C. Calpeptin, an inhibitor of calpain, significantly reduced the number of MVs released after stimulation with 10% NHS and other agonist agents. These agonists in combination also induced the release of MVs significantly compare to singular treatment.

BzATP, ATP, and calcium ionophore (Cal-I) were used to stimulate MV release Cal-I alone did not induce release of MV but in combination with ATP MV release significantly was increased (**Fig.5.2B**).

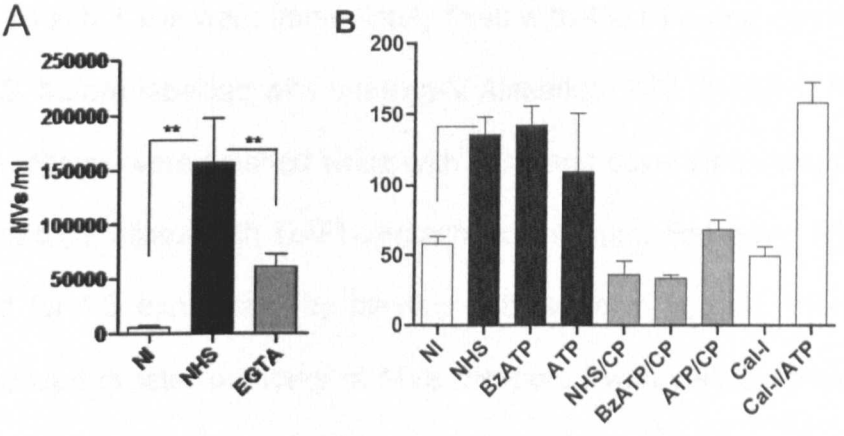

Figure 5.2 MV induction and inhibition using various agents in the presence of NHS. THP-1 cells ($1x10^6$/well) seeded in triplicate into 12-well plates were stimulated at 37°C for 30 min with NHS (10%) alone or in combination with EGTA (5mM).(**A**). Cells treated with BzATP, ATP, Calcium ionophore (Cal-I) induced almost a three-fold increase in MV release compared to non-induced (NI) cells, and the mechanism involved is calcium-dependent, since the Ca^{2+}-chelating agent, EGTA, reduced the number of released vesicles to that of control. Calpeptin (CP) and EGTA inhibited MV release. The release of MVs is inhibited after preincubation (45 min) of cells with calpeptin (inhibitor of calpain, 20 μM). Cells were stimulated by addition of 10% NHS (**B**). In both experiments, MVs were isolated by differential centrifugation at 4,000 *g* for 1 h and a 25,000 *g* 2 h respectively. Values represent the means ± SD of a triplicate experiment. This experiment was repeated three times with similar results. $^{**}P < 0.01$, $^{*}P < 0.05$ were considered statistically significant.

5.2.3 Immunostaining and microscopy analysis of annexin V labelled MVs. HeLa cells ($1x10^5$/well) were seeded overnight into 12-well plates containing 18mm coverslips. After washing twice with PBS, cells were placed into RPMI supplemented with 2 mM $CaCl_2$ and either left untreated, or pretreated with calpeptin and stimulated with NHS (10%v/v) at 37°C with 5% CO_2 for 30 min. Cells were immediately fixed with 4% PFA and washed twice with PBS, before labelling with annexin-V-Alexafluor 488 diluted in PBS with 3% BSA. Plates were washed twice with PBS and coverslips were mounted on microscope slides with DAPI-Vectashield medium. Released MVs were analyzed for PS expression by staining with annexin V; cells treated with NHS released greater numbers of MVs compared with cells pretreated with calpeptin and control cells. These cells were not apoptotic as could be seen by their healthy nucleus, upon staining with DAPI-VECTASHIELD medium (**Fig.5.3**).

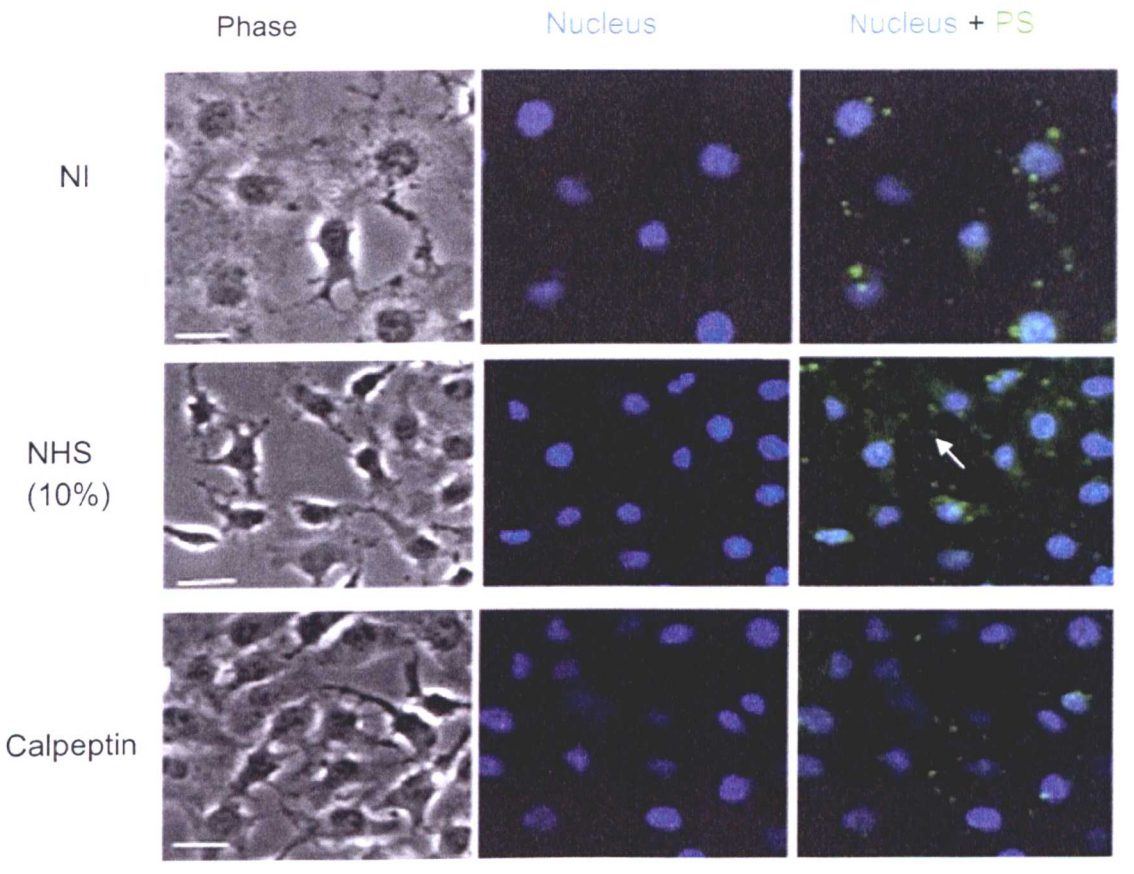

Figure 5.3 Immunofluorescence images of annexin V labelling of MVs in cells induced or inhibited for MV production. Fluorescence microscopic images of MV release, for cells stained with DAPI-VECTASHIELD and annexin V. HeLa cells release MVs observed by immunofluorescence microscopy after labelling with annexin-V Alexafluor 488. PS positive MVs (green dots) are released after stimulating cells with NHS 10% in large numbers compared to non-induced (NI) cells and cells treated with calpeptin. Arrow shows the position of MVs expressing green annexin V relative fluorescence. Scale bar= 100 µm

5.2.4. Depolymerization of the actin cytoskeleton and repair of host cell plasma membrane. MV release is a Ca^{2+}- dependent mechanism which leads to depolymerisation of the actin cytoskeleton and release of MVs[10, 244]. **Fig.5.4A**, demonstrates clearly the presence of depolymerised actin cytoskeleton in HeLa cells treated with NHS (10%), as compared to non-induced cells and cells treated with calpeptin. The presence of LAMP-1 in cells treated with NHS (10%) confirms recruitment of lysosomes to repair the damage caused due to the release of MVs from the plasma membrane. By contrast, MV release was inhibited when cells were pretreated with calpeptin and lysosomes were not recruited to reseal damage (**Fig. 5.4B**).

The release of MVs culminates in loss of plasma membrane integrity. This leads to the influx of Ca^{2+}, which in turn activates the exocytosis of lysosomes to the site of damage. Lysosomes then fuse together and patch-up the damage. This way expression of LAMP-1, a specific lysosomal marker was detected using anti-LAMP-1 antibody and was analysed by fluorescence microscopy.

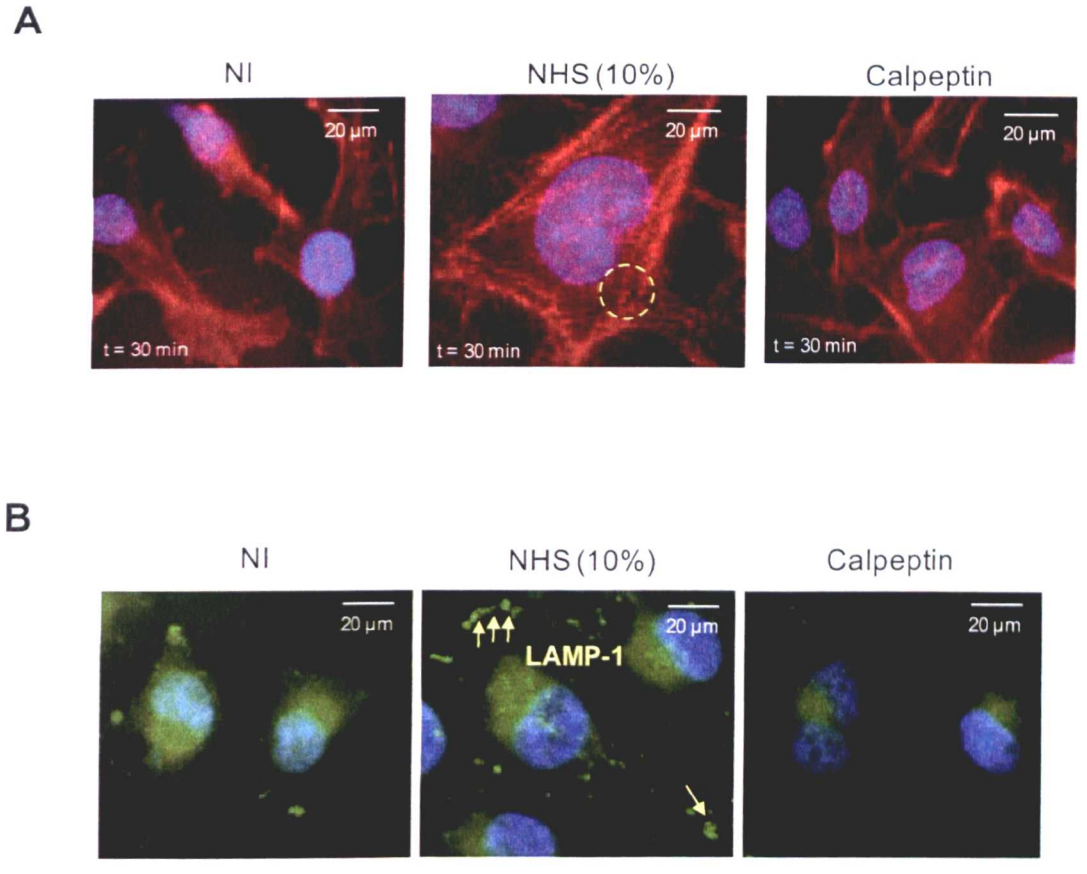

Figure 5.4 MV release is a Ca^{2+}-dependent mechanism leading to disruption of the actin cytoskeleton and increased LAMP-1 expression. Activation of HeLa cells with sublytic complement deposition (10% NHS) and consequent MV release, results in actin rearrangement (visible as punctuate fluorescence after staining with phalloidin AlexaFluor 660) over a 30 min period compared to similarly stimulated cells pre-treated with the calpain inhibitor, calpeptin. In non-induced controls (NI) cells also show comparatively little actin rearrangement (intact actin cytoskeleton) (**A**). Lysosomal glycoprotein LAMP-1 readily appears on the cell surface of HeLa cells in a process strictly dependent on the presence of extracellular Ca^{+2} (**B**). Scale bar= 20 µm.

5.2.5 Normal membrane repair involving lysosomes. The integrity of the membrane is compromised by damage to the cell caused by mechanisms such as the complete detachment of MVs, physical or chemical damage. To investigate the involvement of lysosomes in membrane resealing upon MV release time- lapse experiments were performed. Expression of LAMP-1 in activated HeLa cells reached a maximum up to 30 min after activation compared with non-induced cells and cells treated with calpeptin. Concanavalin A (conA) is an inhibitor of lysosomal exocytosis but does not affect the release of MVs (**Fig.5.5**).

Semiconfluent HeLa cells treated with similar agents were analysed by fluorescence microscopy for expression of annexin-V and of LAMP-1. Stimulation of HeLa cells with 10% NHS causes PM lesion, which results in LAMP-1 translocation to the surface membrane for repair. However, treatment of cells with known inhibitor of lysosomes exocytosis, conA 20μg/ml at room temperature for 10 min with shaking or with calpeptin 20μM for 45 min at 37°C prior to addition of NHS, abrogated lysosomal exocytosis. Cells pre-treated with conA showed uptake of Propidium Iodide (PI) pink labelling due to inhibition of lysosomal migration, which led to an increased PI uptake (**Fig.5.5**). This result agrees with previous findings, which suggested that conA is a specific inhibitor of lysosomal exocytosis. It also confirms that MV production results in damage to the integrity of the host PM which needs to be repaired.

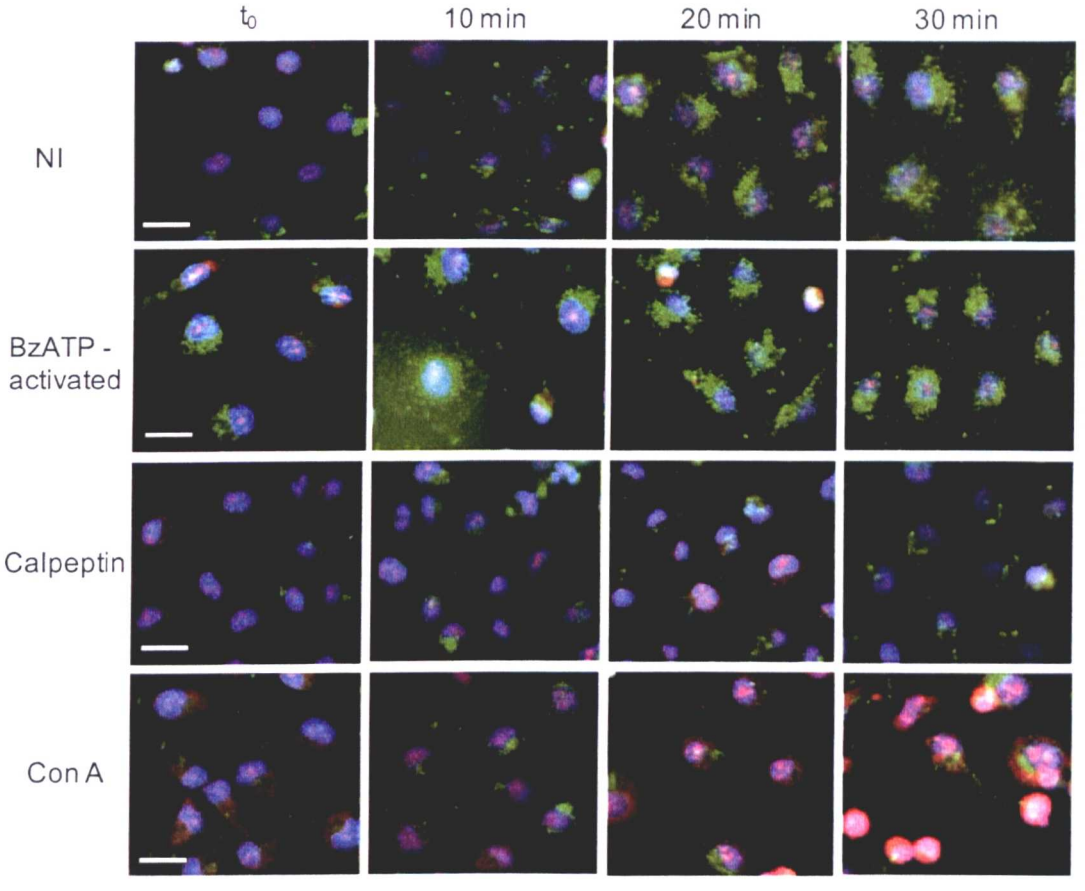

Figure 5.5 MV release is a form of membrane damage, which leads to the recruitment of lysosomes for repair. Stimulation of HeLa cells with BzATP in the absence of calpain inhibitor results in the release of MVs. However, preincubation of HeLa cells with calpeptin, an inhibitor of calpain caused a marked reduction in the release of MVs. Cells pre-treated with calpeptin and conA did not recruit lysosomes compared to activated cells in order to repair membrane damage. Propidium Iodide (PI) (pink) labelling was used to examine membrane repair and staining with DAPI which needs neither fixation nor permeabilisation indicates the nucleus. Cells pre-treated with conA 20µg/ml, RT, 10min showed high PI relative fluorescence (pink colour), which confirms the presence of unrepaired damage in cell membrane. Scale bar = 100 µm

5.2.6 Influx of FM1-43 dye into the cells upon MV release. FM1-43 is a lipophilic fluorescent dye, which is widely used for investigating the mechanisms of activity-dependent vesicle cycling in cell membranes. To confirm whether NHS induction of MVs results in plasma membrane damage that requires repair, HeLa cells were trypsinised into suspension and washed twice with PBS. Cells resuspended in Ringer's solution containing 2 mM $CaCl_2$ and FM1-43 dye (10µg/ml), were seeded into 1.5 ml microcentrifuge tubes in triplicate. Cells were then preincubated or not with concanavalin A (conA) at room temperature for 10 min with shaking, Cytochalasin D (CytD) 10µM at 37°C for 10 min, or with calpeptin and stimulated to microvesiculate by the addition of NHS (10%). EGTA (5mM) was used as a calcium chelator to inhibit Ca^{2+}-dependent microvesiculation.

After treating the cells with NHS (10%) at 37°C for 30 min, the reactions were stopped and fixed with 4% PFA at room temperature for 10 min. Cells were washed twice with PBS and relative FM1-43 fluorescence was measured by flow cytometry. An analysis of the histogram distribution of cells treated with inducing agents showed that when cells were stimulated to release MVs this was perceived by the cells as a signal to initiate membrane repair and to stimulate lysosomal exocytosis. Under such conditions, FM1-43 dye was not internalized because of the intact, repaired plasma membrane. Moreover, CytD an inhibitor of actin polymerization which inhibits MV release resulted in low expression of FM1-43 (**Fig.5.6A** top row).

Cells pretreated with calpeptin induced with NHS also had a low level of FM1-43 influx due to inhibition of microvesiculation. Cells pretreated with con A, an inhibitor of lysosome exocytosis showed high expression of FM 1-43 because of an unrepaired plasma membrane. (**Fig.5.6 A, B**). When cells were treated with EGTA, a calcium chelator, once more inhibiting microvesiculation, the level of FM1-43 staining was low (**Fig.5.6B**) indicating no need for membrane repair. When cells were treated with both conA and EGTA, they expressed more FM1-43 relative fluorescence, which demonstrates diminished lysosomal repair (**Fig.5.6 A** second row, middle histogram).

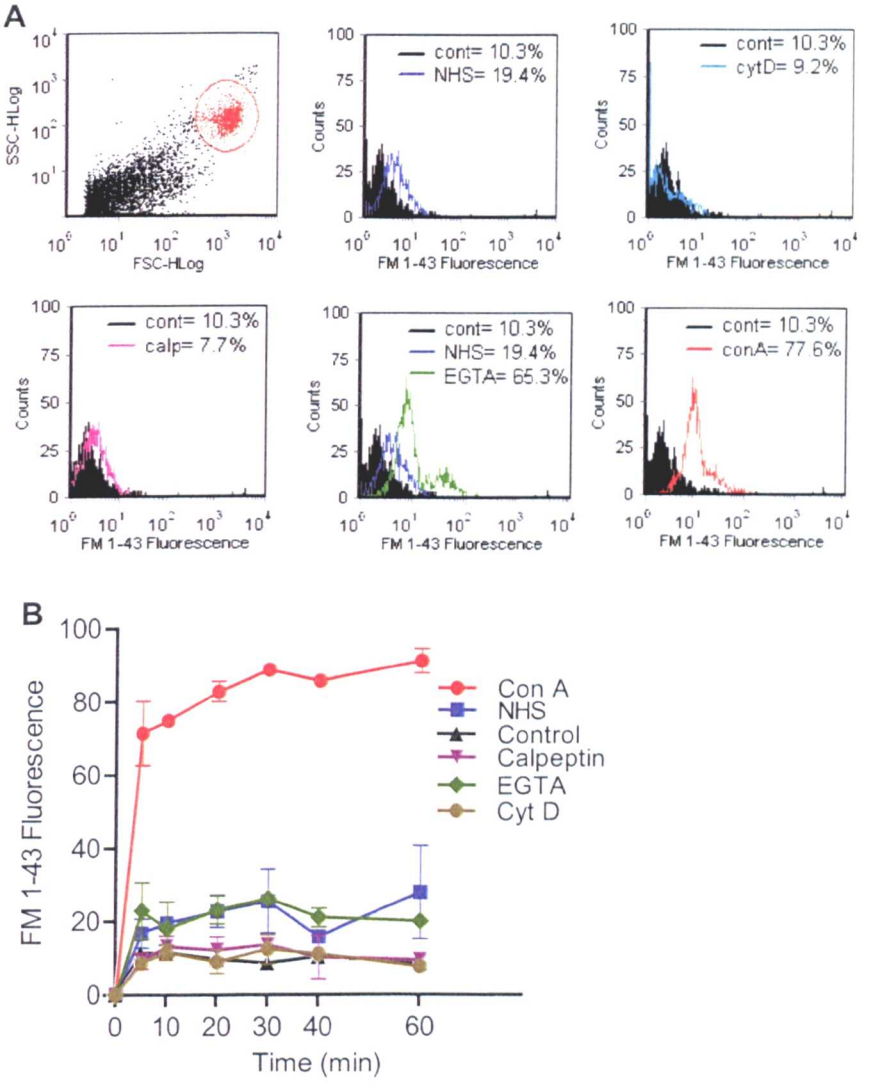

Figure 5.6 Kinetics of membrane repair following the influx of FM 1-43 dye. Labelling experiments using the dye FM-143 also implicated the lysosomal pathway in the repair of damaged plasma membranes of HeLa cells pre-treated with inducing and inhibiting agents (**A**). Time lapse experiments over 60 min were performed on HeLa cells treated with various agents to induce or inhibit MV release. All cells treated with inducing agent released MVs and this release damaged cell membranes, which was repaired by lysosomes, except in cells pretreated with conA in which lysosomal pathway was inhibited, in which case cells expressed significant relative FM1-43 fluorescence (**B**). Data represent 3 independent experiments performed in triplicate. ***P< 0.001, **P< 0.01, *P< 0.05 were considered to be statistically significant.

5.2.7 Evaluation of LAMP-1 and Calcium Green-1AM expression in HeLa cells upon MV release by flow cytometry. To investigate role of LAMP-1 and calcium in membrane resealing, the following experiments were performed. HeLa cells ($5x10^5$/well in triplicate) were trypsinzed, washed and resuspended in Ringer's solution with 2 mM $CaCl_2$ containing Calcium Green-1AM (5 μM) in microcentrifuge tubes. Cells were left unstimulated (NI) or stimulated to microvesiculate with BzATP (300μM) at 37°C, 30min, after preincubating with calpeptin (20 μM), conA (20 μg/ml, RT, 10min) or EGTA (5 mM). Samples were immediately fixed with 4% PFA (10 min, RT) and washed 1X with cold PBS. Samples were labelled with LAMP-1-Alexafluor 488 (5μg/ml). Labelling was performed in 3% BSA/PBS/NaN_3 at 4°C for 1h with shaking. Samples were washed 3x with cold PBS and resuspended in 3% BSA and analyzed by flow cytometry. In addition, after stimulating with BzATP, supernatants were purified and released MVs were quantified.

The data presented (**Fig.5.7A**) demonstrates Calcium Green-1AM expression (63%), indicated by green fluorescence of intracellular Ca^{2+} in HeLa cells treated with inducing agent BzATP. This was maintained in the added presence of conA (71%). By contrast, EGTA and calpeptin inhibited MV release and these cells showed a reduction in Calcium Green-1AM expression. In **Fig.5.7B**, intracellular Ca^{2+} concentration is presented as a bar chart and **Fig.5.7C** shows that release of MVs in cells treated with BzATP and conA was significant compare to non-induced cells. **Fig.5.7D** demonstrates a direct correlation between the concentrations of Calcium

Green-1 AM and anti-LAMP-1-Alexafluor 488 fluorescence expression in cells treated with inducing agents BzATP and conA, and cells treated with calpeptin a calpain inhibitor and EGTA a calcium chelator.

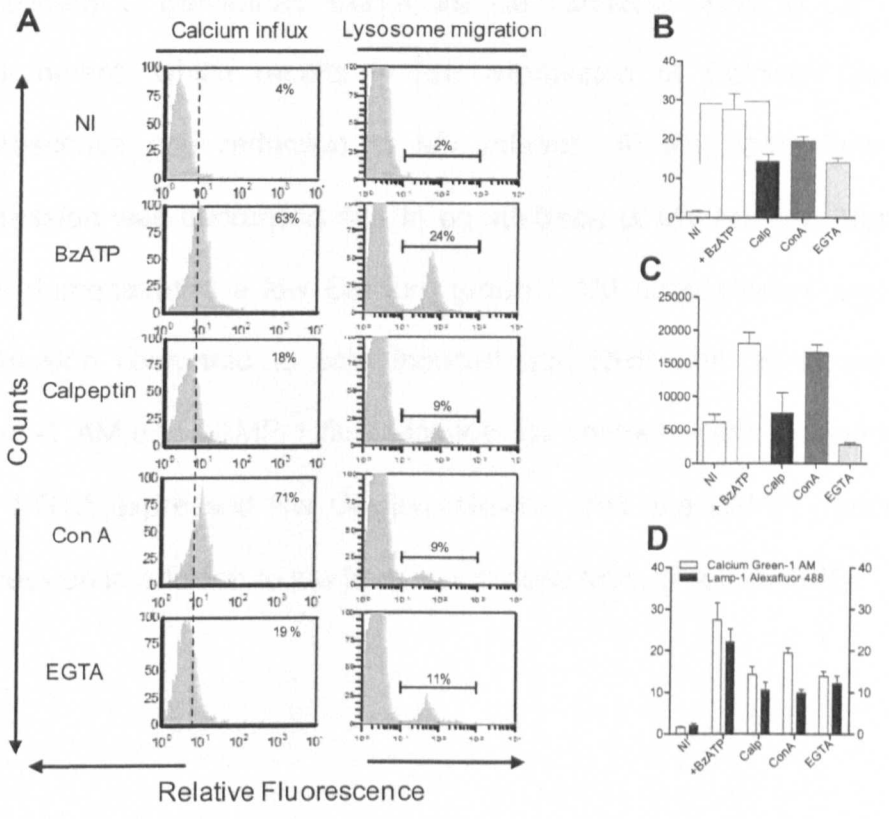

Figure 5.7 MV release and lysosomal exocytosis are both Ca^{2+}-dependent processes. In the absence of inhibitors, BzATP induces the release of MVs compared to non-induced cells (NI). Lysosomes are recruited to the site of damage to reseal breaches (increased green LAMP-1 fluorescence, 24%) so that the plasma membrane remains intact. MV release in the presence of conA continues unabated. However, migration of lysosomes to reseal the resulting damage is abrogated (9% LAMP-1), and in cells treated with EGTA, LAMP-1 decreased which is attributed to the low Ca^{2+} concentration (11%) (**A, B** and **D**); pre-treatment of HeLa cells ($5x10^5$/well in triplicate) with calpeptin (20 μM) inhibits MV release in contrast with BzATP which induced microvesiculation (**C**). A persistent decrease of fluorescent intensity (as an indicator of dye loss) indicates resealing failure. This data implies that inhibition of MV release means less damage is sustained by plasma membrane which results in fewer lysosomes being required for resealing, as host cell PM integrity is intact. Data represent 3 independent experiments performed in triplicate. ***$P< 0.001$, **$P< 0.01$, *$P< 0.05$ were considered to be statistically significant.

5.2.8 Quantitative analyses of LAMP-1 and Calcium Green-AM fluorescence in HeLa cells treated with inducing or inhibiting agents. Cell activation by NHS (10%) also results in a Ca^{2+} influx, MV release, and recruitment of lysosomes. EGTA, the Ca^{2+} chelator removes Ca^{2+} from the environment, which results in low expression of Calcium green-1 AM fluorescence and reduction of MV release. At the same time LAMP-1 expression was decreased due to an inhibition of MV release. Non-induced cells demonstrated a low Calcium green-1 AM concentration and LAMP-1 expression compared to cells induced with NHS with significant Calcium Green-1 AM and LAMP-1 fluorescence. By contrast, cells treated with NHS and EGTA expressed low Calcium Green-1 AM and LAMP-1 fluorescence expression in addition to low level of released MVs. (**Fig.5.8. A-D**).

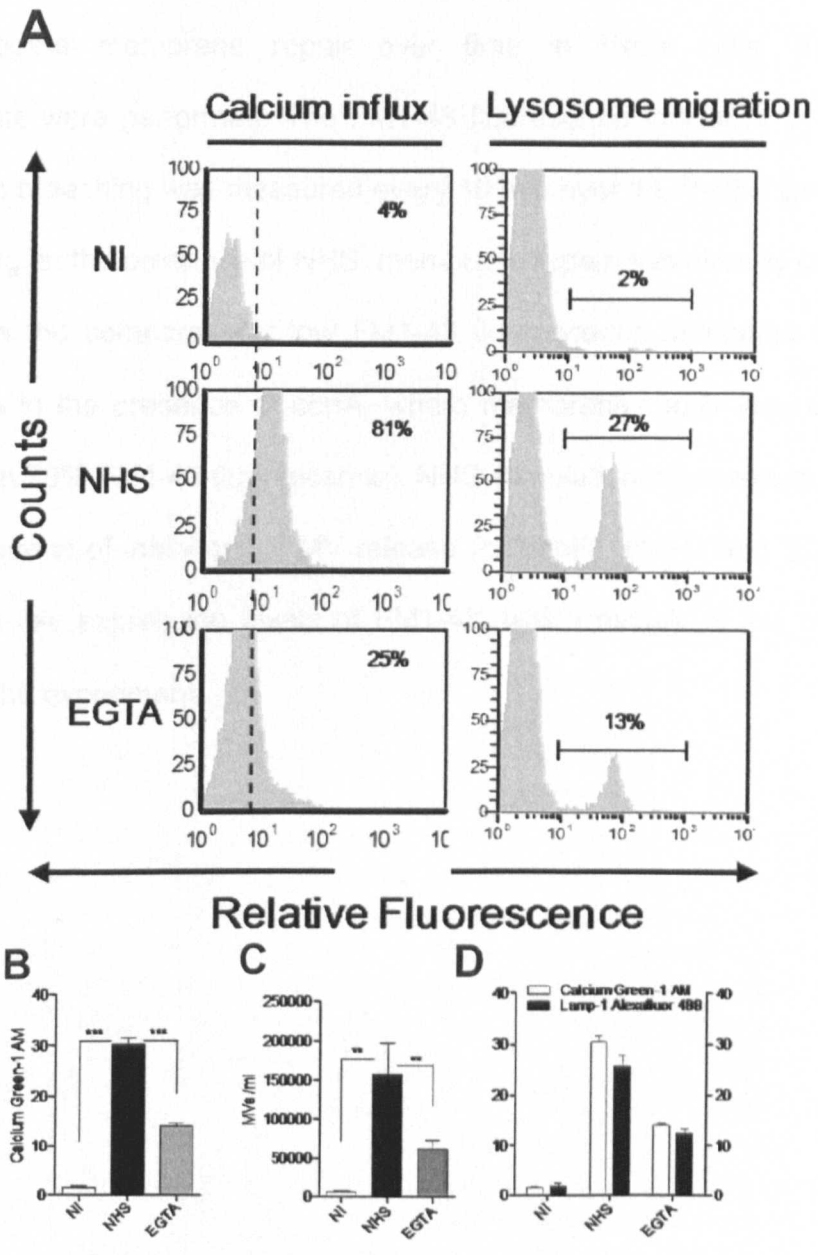

Figure 5.8 Inhibition of MV release modulates lysosomal exocytosis and repair. In cells treated with EGTA, LAMP-1 fluorescence decreased which is attributed to the low Ca^{2+} concentration (**A, B and D**). Lysosome mediated plasma membrane repair is compromised in cells pre-treated with conA. MV release was inhibited in cells treated with EGTA but induced in cells treated with NHS (**C**). Data represent 3 independent experiments performed in triplicate. ***P< 0.001, **P< 0.01, *P< 0.05 were considered to be statistically significant.

5.2.9 Influx of FM1-43 dye in time-lapse experiments.

To investigate membrane repair over time in HeLa cells, the next experiments were performed. The FM1-43 fluoresence indicative of plasma membrane breaching was measured every 10 min over 1h. By the time of the first reading, in the presence of NHS, membrane repair was already complete as seen in the comparatively low FM1-43 fluorescence compared to NHS stimulation in the presence of conA, where membrane repair was inhibited (resulting in 79% FM1-43 fluorsecence). NHS stimulation of microvesiculation in the presence of inhibitors of MV release (calpeptin, Cyt D and EGTA) all resulted in low expression levels of FM1-43, which remained low over the course of the experiment.

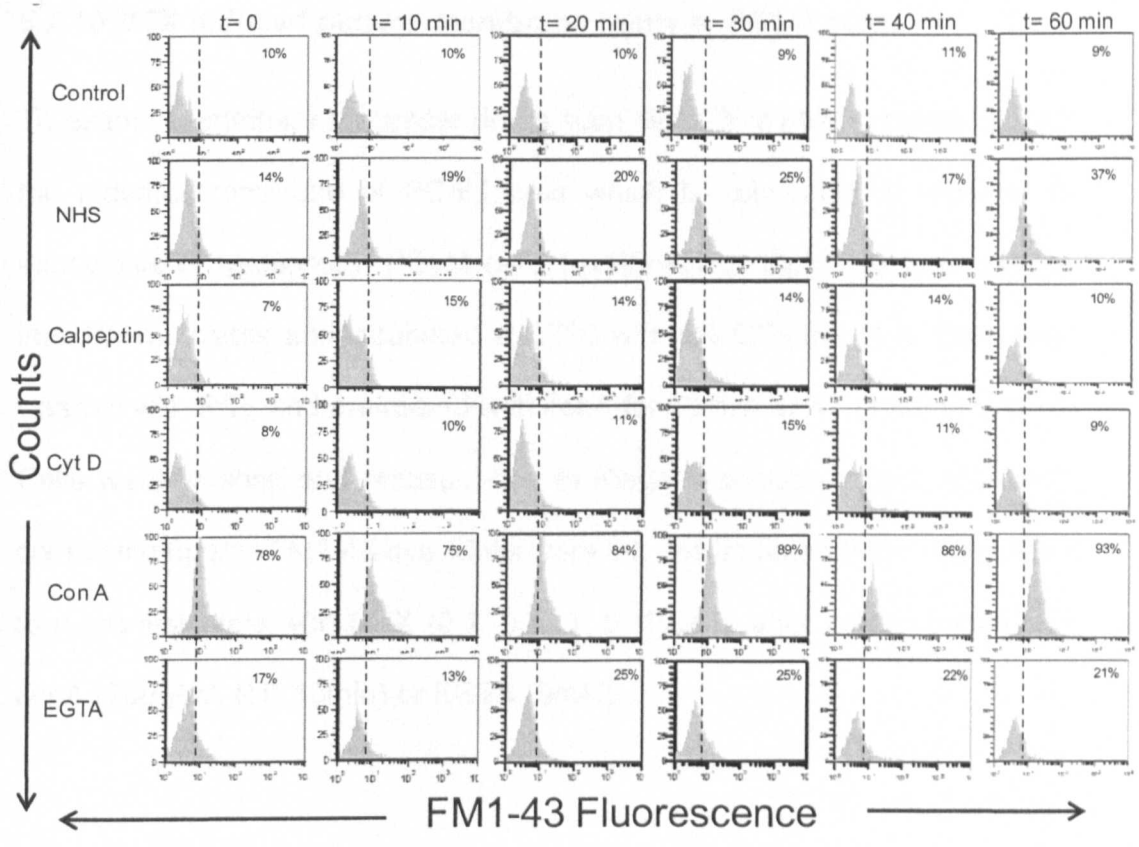

Figure 5.9 Induction of MV release is considered damage to cell membrane and leads to lysosomal recruitment. FM1-43 loaded HeLa cells were subjected to time lapse fluorescence measuring. HeLa cells treated with either inducing or inhibiting agents expressed high or low levels of FM1-43 fluorescence comparing to non-induced cells in time-laps experiments. Cells pre-treated with calpeptin and con A did not recruit lysosomes compared to activated cells in order to repair membrane damage. FM1-43 labelling was used to examine membrane repair. An uptake of FM1-43 indicates a damage in plasma membrane. Cells pre-treated with conA showed FM1-43 relative fluorescence, which confirms the presence of unrepaired damage in cell membrane. Data represent two independent experiments performed in triplicate. ***$P< 0.001$, **$P< 0.01$, *$P< 0.05$ were considered to be statistically significant.

5.2.10 MTX induced plasma membrane injury in PC3M cells.

To examine whether anti-cancer drugs such as MTX is able to cause injury to the plasma membrane of PC3M cells which is subsequently repaired by exocytosis of lysosomes, PC3M cells ($5x10^5$/well in triplicate) were seeded into 12-well plates and incubated at 37°C with 5% CO_2 for 24 h. Cells were washed with PBS and pretreated with conA for 10 min at RT or left untreated. Cells were washed and resuspended in Ringer's solution with 2 mM $CaCl_2$ containing 5µg/ml FM1-43 dye. Cells were left unstimulated (NI) or stimulated to microvesiculate with MTX (0.1, 0.5, 1, 5, 10µM), after preincubation with conA (20µg/ml, RT, 10min) or EGTA (5mM).

Plates were immediately assessed for relative FM1-43 fluorescence using a FLUOstar Omega microplate reader over 30 min. Cells pretreated with conA and with MTX expressed relatively high fluorescence for FM1-43 compared to cells treated with MTX only. These results demonstrate that when MVs are released due to MTX induction the plasma membrane is damaged upon MV release. Lysosomes are able to repair this damage but when lysosomal recruitment is inhibited with conA, significant influx of FM1-43 dye inside the cells is detectable in a dose-dependent response (**Fig. 5.10 A-E**).

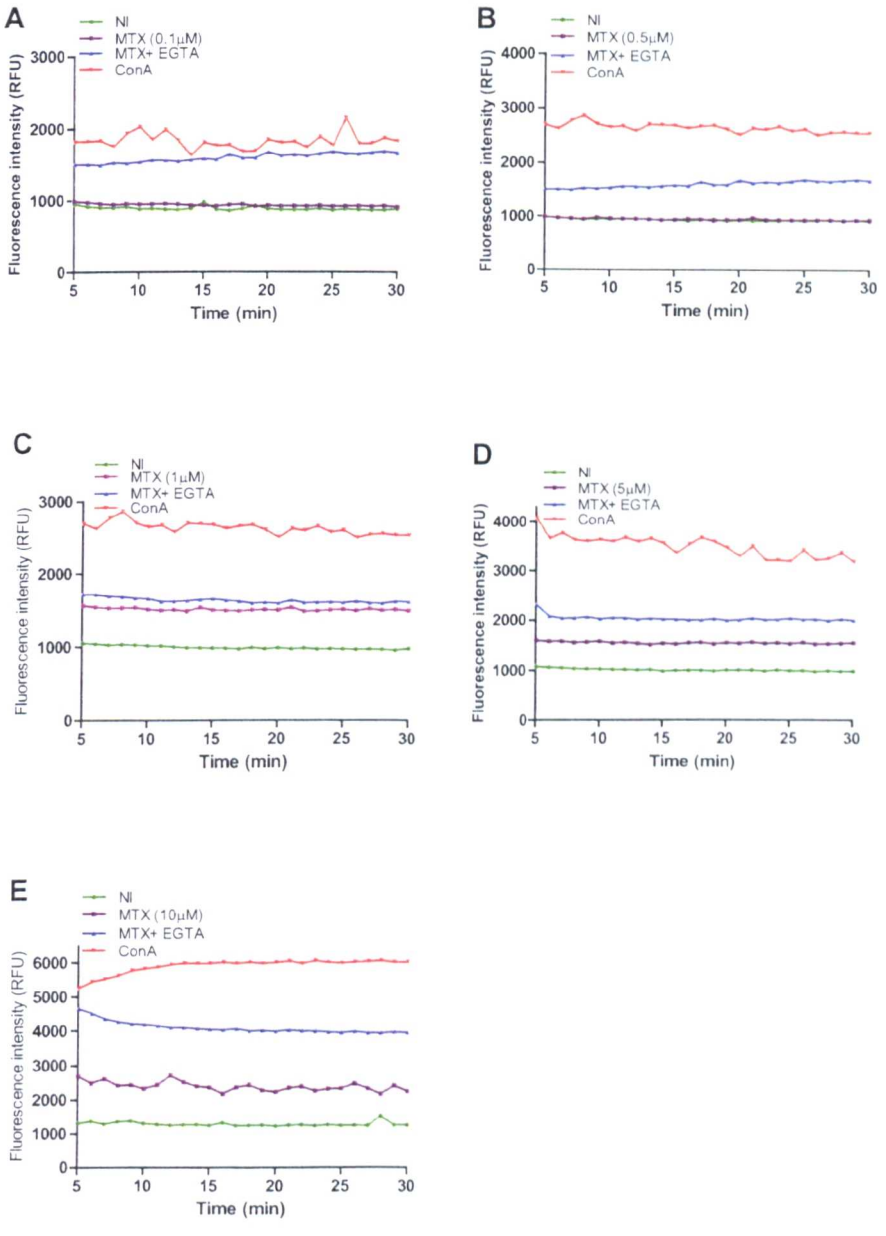

Figure 5.10 Concanavalin A treatment inhibits membrane repair in PC3M cells. Cells which were pre-treated with conA before application of MTX, expressed significant levels of FM1-43 fluorescence over 30 min in a (MTX-) dose-dependent manner (**A-E**). In addition these results emphasise that low concentration of drug induce MV release, thereby recruiting lysosomes to the cell surface. Control cells efficiently resealed their plasma membrane in the presence of Ca^{2+}, effectively blocking the influx of FM1-43 uptake. By contrast, cells were defective in controlling the intracellular flow of FM1-43 when treated with EGTA and conA and failed to reseal their plasma membrane and therefore stopping influx of FM1-43. Data represent two independent experiments performed in triplicate. ***$P<$ 0.001, **$P<$ 0.01, *$P<$ 0.05 were considered to be statistically significant.

5.2.11 Concomitant treatment of HeLa cells with Doc and conA results in an inhibition of plasma membrane repair. To examine the effect of Doc treated PC3M cells on membrane repair, in the presence or absence of con A, the same set of experiments as in section 5.2.10 were performed but this time cells were treated with various concentrations of Doc instead. Release of MVs in the presence of conA continued unabated. However, migration of lysosomes to reseal the resulting damage was abrogated, thus, leading to an increase in FM1-43 dye influx into the cells. EGTA treatment reduced the release of MVs and also inhibited the migration of lysosomes. The release of MVs is directly related to lysosomal exocytosis. This means that more lysosomes are recruited to the surface for repair, when cells endure more damage due to release of MVs. If the process of migration of lysosomes to plug any breaches to the integrity plasma membrane was inhibited by conA the resulting influx of FM1-43 dye was thereby increased (**Fig. 5.11A-E**).

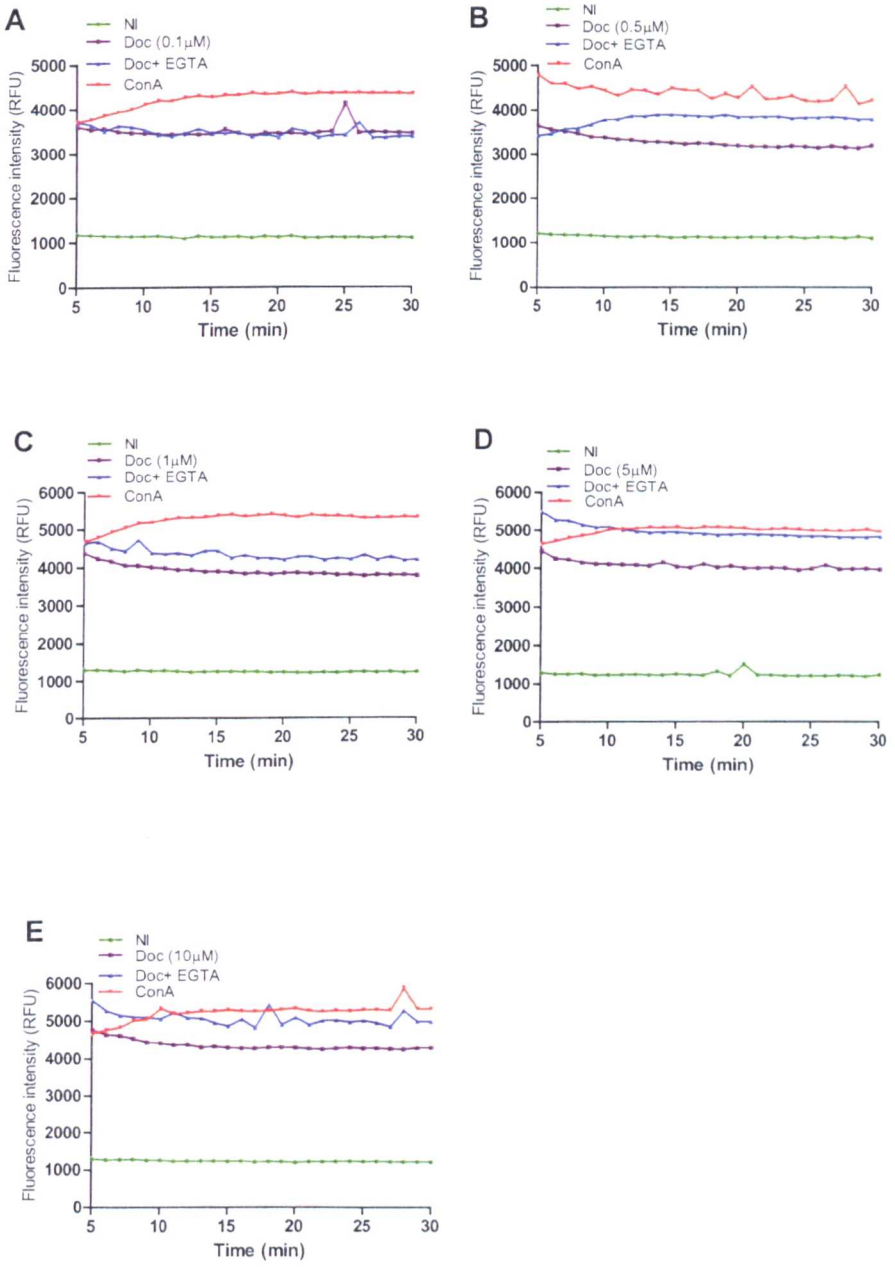

Figure 5.11 ConA inhibits lysosomal exocytosis and repair in Doc treated PC3M cells. Semiconfluent PC3M cells pretreated with both conA and Doc expressed high levels of FM1-43 fluorescence. By contrast, FM1-43 fluorescence was diminished in cells treated in combination with Doc and EGTA (**A-E**). Once more this implies that exocytosis of lysosomes is a Ca^{2+}-dependent process. Data represent two independent experiments performed in triplicate. ***P< 0.001, **P< 0.01, *P< 0.05 were considered to be statistically significant.

5.3 Discussion

Early studies demonstrated that wounded eukaryotic cells in sea urchin eggs rapidly repair their plasma membrane by a Ca^{2+}-dependent mechanism[208]. Several decades later, for the first time a functional link between plasma membrane repair and fusion of lysosomal organelles occurring in less than 30 seconds was established[245]. This process is a Ca^{2+}-dependent exocytosis of lysosoms which forms a patch that directly fuses with damaged membrane[209].

In this study, I investigated the correlation between microvesicle release and Ca^{2+}-dependent exocytosis of lysosoms which is responsible for plasma membrane repair. I provide experimental evidence supporting the conclusion that MV release is a Ca^{2+}-dependent process leading to disruption of the actin cytoskeleton and considered as a type of damage into the plasma membrane. The inhibition of exocytosis of lysosoms to the site of damage impairs the ability of cells to response to injury and loss of PM integrity which results in cell death (**Fig.5.4A, B, 5.5**).

In the present study several cell types were stimulated with NHS or anticancer drugs and as a result cells released microvesicles (**Figs.5.2A, B, Fig.5.7C, Fig.5.10A-E**). These vesicles were positive for exposure of phosphatidylserine after staining with annexin V (**Fig.5.3**). These results showed that induction of microvesicles is Ca^{2+}-dependent process and

calcium chelator, such as EGTA was able to abrogate release of MVs (**Fig.5.8**). This work also showed for the first time that MV release itself causes damage to the cell membrane which is repaired by lysosoms (**Fig.5.7**). Furthermore, inhibition of lysosomal exocytosis using agents such as conA which inhibit PM repair led to cell death (**Fig.5.5**). Interestingly, blocking lysosomal exocytosis however failed to inhibit MV release after stimulating with sublytic complement. These results also showed that MV release is inhibited by calpeptin an inhibitor of calpain (**Fig.5.7C**). Together, data presented demonstrate that MV release is Ca^{2+}-dependent, since their release can be inhibited by calpeptin and EGTA (**Figs.5.7A, B,C, 5.8C**).

It has been shown before that stress factors and pore-forming complexes cause damage of the plasma membrane which is rapidly resealed by lysosomes when they fuse together and patch-up the damage[246]. Rapid repair is crucial in order to prevent loss of cell contents. When cells were stimulated with NHS or anticancer drugs higher numbers of MVs were released and in the absence of inhibitors, increased fluorescence of the lysosome-specific marker, LAMP-1 was detected (**Figs.5.4B,5.7A,D,5.9**). An elevated level of LAMP-1 fluorescence shows activation of lysosomal repair. As aforementioned, release of MVs caused damage to the plasma membrane and so preventing the release of MVs should result in less damage to PM and reduced LAMP-1 fluorescence. My data confirmed that cells treated with calpeptin failed to shed MVs after induction with NHS or anticancer drugs. In addition, decreased level of LAMP-1 fluorescence was

detected (**Fig.5.7A, D, 5.9**). Cells treated with conA showed reduction in LAMP-1 fluorescence which indicates inhibition of lysosomal exocytosis (**Fig.5.7A, C, and D**). However, conA did not inhibit MV release since number of MVs released upon induction remained unabated compared to untreated control cells (**Fig.5.7C**).

Moreover, my data confirmed that treatment of the cells (RT for 10 min) with conA resulted in influx of FM1-43 dye or PI into the cells post MV induction (**Fig.5.5, 5.6A, B, 5.9**). Conversely, MV release was significantly reduced in cells treated with calpeptin and absence of MV release showed no damage was sustained by the host plasma membrane (**Figs.5.9, 5.10A-E, 5.11A-E**). This also affirms that MV release is an independent mechanism from lysosomal exocytosis as MVs are released after blocking with conA. This confirms that Ca^{2+}-dependent MV release resulted in lysosomal exocytosis (**Fig.5.9**).

Fluorescence microscopy results showed that release of MVs induced by NHS resulted in the recruitment of lysosomes which in turn repair the resultant damage so that less PI was incorporated. Moreover, calpeptin inhibited MV release and PI uptake was less indicative of a lake of damage (**Fig.5.5**). Interestingly, MV release remained unabated in the presence of conA which unlike calpeptin does not inhibit MV release.

This confirms that although lysosomal exocytosis was abolished by conA but MV release is not dependent on lysosomes. To rapidly repair the plasma membrane upon MV release lysosomal exocytosis to the site of damage is vital for halting loss of host cell integrity (**Fig.5.6, 5.7A, C**).

Finally, MVs have been characterised in recent years and their role in intercellular communication have been well reported[6, 70]. In this study, I tested the hypothesis that tumour cells when challenged with anti-cancer agents induce resistance by riding themselves of the agonists via transfer of the molecules to their surface and releasing them inside MVs. The resulting plasma membrane damage caused by the release of MVs from the tumour cell surface is subsequently repaired by lysosomes recruited to the surface membrane. This mechanism unravels a novel role for MVs in anti-cancer drug resistance and plasma membrane repair mechanisms.

My *in vitro* findings show that MV production in response to stress or insertion of the membrane attack complex (MAC) causes lesions on the surface of the host cell plasma membrane, which if left unrepaired may lead to loss of cytoplasmic contents and cell death. I show that MV release is a form of membrane damage, which must be resealed immediately using, activated lysosomes if the host cell is to survive and proliferate.

6. General Discussion

6. Discussion

6.1 The composition and function of MVs

Microvesicles are small (0.1-≤1 μm in diameter) heterogeneous vesicles released from cells in the resting state or upon activation[1]. Their number is increased upon cellular activation or in cancer. MVs are released in a Ca^{2+}-dependent process in which calpain has a crucial role in regulating the depolymerization of the actin cytoskeleton[4, 244].

Once released, these vesicles carry mRNA, miRNA, as well as a wide range of cytokines, chemokines and acute phase proteins and participate in mediating intercellular communication[5, 11, 70]. MVs play important roles in modulating the host immune system, as well as influencing host disease pathogenesis and cell signalling[5, 10, 18]. In this study MVs were isolated by various methods including filtration, and differential centrifugation. MV isolation and analysis from biological samples has the potential to provide information about the state and progression of malignant tumours and their appearance is unpredictable during metastases[123].

MVs have helped shift our understanding of the pathophysiological mechanisms associated with several diseases, and their detection may be crucial in providing information about the pathology of specific diseases[13, 247]. This is significant in tissues such as the central nervous system which is not directly accessible for examination[7]. The detection of chronically elevated levels of circulating MVs in patients with sickle cell disease provides an

insight into the chronic endothelial attack that characterises this condition, and may provide an important tool in measuring the protective effects of therapeutic interventions in an early and non-invasive manner[24]. An increase in intracellular calcium levels, culminates in rearrangement of the actin cytoskeleton and leads to the release of MVs from the host plasma membrane (PM) by exocytic budding[30]. As aforementioned, MVs play important roles in crosstalk communication and they transfer, and regulate processes such as apoptosis, inflammation, coagulation and proliferation between cells[27]. As a result, MVs have been well documented to play important roles in various diseases[18, 119]. The mechanism leading to the release of MVs is initiated by increase in cytosolic Ca^{2+}, which in turn activates the enzyme, calpain, involved in detachment of vesicles from the parent cell[248]. Inhibition of MV release with EGTA confirmed the involvement of calcium in the process. Similarly, the calpain inhibitor, calpeptin, abolished the release of MVs after stimulation of cells with either Normal Human Serum (NHS) or anti-cancer drugs.

Normal human serum was initially used to stimulate the release MVs from cells due to the presence of complement factors such as C5b-9 Membrane attack complex (MAC)[25]. MVs released into the conditioned medium upon stimulation with NHS, were isolated by differential centrifugation, starting with 4,000 *g* for 1 h and subsequent centrifugation at 25,000 *g* for 2 h at 4°C.

MV release in pathological and physiological conditions such as cell activation, cell injury, apoptosis and necrosis leads to loss of cell asymmetry

and exposure of PS on the outer cell leaflet. In blood platelets, PS exposure on the outer layer of the cell membrane may in turn result in a prothrombotic state[30].

6.2 The mechanism of MV release

The mechanism of MV release described here is unique and differs from the release of other membrane vesicles such as exosomes and apoptotic bodies. Apoptosis, or programmed cell death, is a process which occurs after cellular damage. It results in functional and physiological changes in the nucleus, cytoplasm and cell membrane[28].

Cells shrink and vesicles and vacuoles are formed. Chromatin condenses and the degraded nucleus fragments. The cell forms apoptotic bodies, which express PS on their surface membrane rendering them susceptible to phagocytosis by macrophages. Apoptotic bodies are larger in diameter and contain fragmented DNA[47], in contrast to MVs, which are smaller and contain intact DNA. Finally, the release of MVs is distinct from both apoptotic bodies and exosomes[46].

In the resting state, cell membrane is asymmetric depending on the composition and the distribution of phospholipids in the plasma membrane. Phosphatidylcholine and sphingomyelin are located in the outer leaflet, while phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE) are present in the inner layer[28]. This plasma membrane asymmetry is regulated by

transmembrane enzymes, which promote transverse migration of anionic phospholipids such as PS from the inner to outer leaflet of the plasma membrane prior to the release of MVs. Any cellular event that leads to the loss of cell asymmetry mostly results in MV release[38]. The cytoskeleton is therefore disrupted, resulting in membrane budding and microvesicle shedding[244].

6.3 MV involvement in the spread of CVB1 infection

The first hypothesis examined in this thesis was whether MVs play a significant role during viral infection. Previous studies have shown that Coxsackievirus B1 (CVB1) a member of the *Picornaviridae* family is associated with myocarditis. Like most non-enveloped, single-stranded RNA viruses, CVB1 is well established to cause infection via the lytic mode of infection, which requires contact between the virus and the target cell plasma membrane [222]. However, although recent reports have described several non-lytic virus release mechanisms by which virus particles are transmitted to secondary sites of infection, these processes still remain unclear[140].

Many viruses cross host endothelial barriers to intrude and spread to new sites. CVBs bind to DAF and CAR receptors and activate specific signalling molecules and enter host cells[142, 232]. It is well documented that in addition to the lytic mechanism of infection, other strategies are involved in the spread of viral progeny to secondary sites of infection[152, 153]. A new study illustrated involvement of a non-lytic cell-cell mechanism in CVB3 infection [130].

However, so far there is very little data to support this mechanism in the literature and so the rapid dissemination of virus particles to secondary sites of infection still remains an unresolved puzzle. This study showed for the first time that infection of HeLa cells by CVB1 culminates in apoptosis and release of virus-induced apoptotic microvesicles (vaMVs) with CVB1 virions encapsulated in them. These vaMVs carried and disseminated CVB1 virions through a non-lytic MV-to-cell mode to target non-infected HeLa cells. Moreover, In agreement with earlier work[25], vaMVs carry and transfer apoptotic signals and death molecules such as caspase-3 and Fas to recipient cells inducing further cell death. These vaMVs can also induce production of more MVs, which is abrogated upon inhibition of calpain activation with calpeptin.

Immunofluorescence microscopic images showed that co-culturing healthy HeLa cells with vaMVs resulted in detection of CVB1 virions in cells. Immunoblotting with anti-mouse CVB1-antibody further confirmed the presence of CVB1 proteins in vaMVs lysates. Notably, knocking down *CAPNS1* by siRNA in HeLa cells led to inhibition of MV release suggesting that MVs mediated the transfer of apoptotic signals to secondary sites of infection.

Together, vaMVs could act as a yet unknown non-lytic MV-to-cell mode of transmission, manipulated by non-enveloped viruses, so further confirming the role of MVs as novel tools of intercellular communication. The first studies

in this area showed that platelet-derived MVs transfer tissue factor to monocytes so inducing coagulation[176]. In this vein, CMIRC previously reported an unconventional secretory pathway by involving the release of MVs, whereby molecules lacking an N-terminal sequence are transported to the extracellular matrix[172].

My results offer an explanation to the rapid dissemination of virus particles to neighbouring cells during CVB1 infection, and provide compelling evidence to explain the elevated induction of apoptosis detected in neighbouring cells during CVB1 infection. I propose a novel non-lytic MV-to-cell viral spread mechanism by which CVB1 efficiently reaches new target cells in contrast to the lytic spread of infection. This mechanism offers a unique advantage to the virus, in the sense that, virus particles encapsulated within vaMVs would also be protected against host-mediated immune surveillance. Finally, due to their size (0.1-≤1 μm diameter), vaMVs are likely to disseminate further to distant regions and to evade immune attack. Thus, the ability of vaMVs to bring viruses closer to several cell types contributes to viral infection.

6.4 Role of MVs in cancer drug resistance

Previously, it has been shown that cancer cells release MVs excessively compared to healthy cells and that these MVs are involved in angiogenesis, metastasis and escape from the immune system[131]. Moreover, tumour derived-MVs carrying metalloproteases contribute to tumour invasion and metastasis[18]. The development of insensitivity to a wide range of

chemotherapeutic agents occurs during cancer therapy leading to multi-drug resistance (MDR). However, the process used by cancer cells to avoid anticancer drug-induced apoptosis remains indefinite[236]. Earlier reports have suggested involvement of P-gp, MRP and BCRP, which are members of the ATP–binding cassette transporter family, participating in the active efflux of a broad range of anticancer drugs via the cell membrane[249].

The involvement of MV release as a non-genetic mechanism for the development of MDR was reported for the first time by Bebawy M et al (2009). They showed that MVs transferred P-gp from MDR leukaemic cells to drug-sensitive target cells leading to drug insensitivity in the target cells[132]. In this study, a new hypothesis was suggested to examine whether MVs released by cancer cells after treatment with chemotherapeutic agents, carry the agonists and whether the inhibition of MV release could offer better prognosis in treating MDR-related conditions.

My data show that anticancer drugs such as 5-FU, MTX, and Doc, induce release of MVs. The involvement of calpain in MV release has been demonstrated in previous studies[34]. Here I demonstrated that anticancer drugs such as methotrexate and docetaxel function synergistically with calpeptin to induce effective apoptosis in target cancer cells even when administered at lower doses (100-fold less). This suggests a novel mechanism that might be considered for cancer therapeutics. My data confirmed that the release of MVs acts as a mechanism leading to efflux of

drugs from the cell surface of cancer cells and contributes to drug resistance. Inhibition of MV shedding by calpeptin or silencing *CAPNS1* in drug treated cancer cells increases apoptosis. The *in vivo* results further confirmed that an expulsion of therapeutic drugs from tumour cells through shedding of MVs culminated in tumour-cell survival and tumour sizes were reduced upon treatment with a combination of drug and calpeptin.

6.5 MV release leads to exocytosis of lysosomes and plasma membrane repair

Restoration of plasma membrane integrity after injury is crucial for eukaryotic cell survival. This repair mechanism is a Ca^{2+}-regulated exocytosis of lysosomes leading to the formation of a patch which fuses directly to the site of damage[205]. Data presented in this study are in agreement, but also illustrate that this elevated cytosolic Ca^{2+} resulted in the release of MVs which activates migration of specific organelles, including lysosomes to the site of damage. I provide experimental evidence supporting the conclusion that MV release is a Ca^{2+}-dependent process leading to disruption of the actin cytoskeleton and perceived as a type of damage to the plasma membrane. These data showed that when agents such as, EGTA, a calcium chelator is used, the release of MVs is abrogated. This work showed for the first time that MV release causes a momentary and minute damage to the cell membrane which is repaired by lysosomes. Together, my data demonstrate that rapid repair is crucial in order to prevent loss of cell contents. Cells stimulated with NHS or anticancer drugs released higher number of MVs and increased fluorescence of the lysosome-specific marker,

LAMP-1, thus indicating activation of lysosomal repair. Conversely, the inhibition of exocytosis of lysosomes to the site of damage which was detected by reduced LAMP-1 expression impairs the ability of cells to responsed to injury leading to the loss of PM integrity and cell death.

6.6 Conclusion

In summary, microvesicles released from the plasma membrane expressing surface phosphatidylserine and ranging from 0.1-≤1μm in diameter carry various membrane proteins, lipids and cytoplasmic components characteristic of the parental cell[4, 10]. The significant role of MVs has been emphasised in diseases such as diabetes mellitus, rheumatoid arthritis, sickle cell anaemia, thrombosis, cancers and in angiogenesis[48, 123, 165]. MVs involvement in receptor transfer shed light on host cell invasion mechanisms such as HIV and prions[250]. The work presented in this thesis further demonstrates the significant role of these vesicles in viral infection, cancer drug resistance, and plasma membrane resealing. This thesis provides evidence that release of MVs acts as a mechanism of MV-to-cell transmission of CVB1 virions. Calpain activation is essential for both CVB1 entry and replication. Using either small interfering RNA (siRNA) to knockdown calpain expression or calpeptin to inhibit calpain, leads to reduction of MV release and this reduction abolishes CVB1 entry and spread in HeLa cells. Furthermore, this study, having postulated that MV shedding is associated with anti-cancer drug resistance set about to investigate whether targeting the disruption of calpain, would reduce multi-drug resistance in

cancer cells. As a result, a correlation between vesicle-shedding and chemoresistance showed that MV release indeed serves as a mechanism of drug expulsion in the prostate cancer cell line, PC3M. These data demonstrated that *in vitro* inhibition of MV release with calpeptin significantly increased anticancer drug-mediated apoptosis and in an *in vivo* xenograft mouse model of prostate cancer, 100-fold lower concentrations of MTX or Doc achieved the same reductions in tumour size when calpeptin was synergistically injected with MTX or Doc.

This study has expanded the knowledge of microvesiculation further through testing a new hypothesis that MV release, although vital for cell survival and intercellular communication, may constitute a form of monentary damage in the plasma membrane which requires rapid repair. In addition, it was found that microvesiculation led to the recruitment of lysosomes to the site of damage for the purpose of initiating a repair by plugging the membrane break as was confirmed by the expression of LAMP-1 fluorescence. However, inhibition of calpain by calpeptin abrogated MV release and also LAMP-1 expression. Moreover, inhibition of lysosomal migration had no effect on MV release but resulted in decreased expression of LAMP-1 fluorescence suggesting that lysosomal recruitment was inhibited. Finally, given the systemic nature of MVs, further studies are required to identify their therapeutic significance as they could potentially be used diagnostically to predict therapeutic response and guide treatment strategies in cancer patients.

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Appendix

1. Reagents and Solutions

Acrylamide/Bisacrylamide	Sigma-Aldrich
Annexin V Alexa Fluor 488	Invitrogen
Annexin V reagent	R&D Systems
APS (Ammonium persulphate)	Sigma-Aldrich
Bacitracin	Sigma-Aldrich
Barbituric acid	Sigma-Aldrich
BCA protein assay kit	Pierce Biosciences
BSA (Bovine serum albumin)	Sigma-Aldrich
BDMA (Benzyl dimethylamine)	Agar Scientific
Bromophenol blue	Fisher Scientific
Calcium AM	Invitrogen
Calcium chloride	Sigma-Aldrich
Calpeptin	Merck Biosciences
Chlorpromazine	Sigma-Aldrich
Concanavalin A	Sigma-Aldrich
Coomassie brilliant blue	BDH Limited, Poole, England
Cytochalacin D	eBiosciences
DAPI-VECTASHIELD	Vector Laboratories Inc, CA, USA
DDSA (Dodecenyl succinic anhydride)	Agar Scientific
DEAE-Cellulose	Sigma-Aldrich
DMSO (Dimethyl sulfoxide)	Fisher Scientific
Docetoxel	Sigma-Aldrich
DDT (Dithiol threitol)	SignaGen Laboratories
ECL WB Detection Reagent	Sigma-Aldrich
EGTA	Kind gift from IBCHN
Equilibration buffer	Fisher Scientific

Ethanol	Sigma-Aldrich
BS (Foetal Bovine Serum)	Sigma-Aldrich
Fix and Perm Cell Permrabilisation	ADG, Germany
5-Flurouracil	Sigma-Aldrich
FM1-43	Invirtogen
Glucose	Agar Scientific
Glutaraldehyde	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
Guava ViaCount Reagent	Guava Technologies, Uk
Guava Nexine Reagent	Guava Technologies, Uk
Halt Protease Cocktail	Pierce, Thermo-Scientific
HCL (Hydrochloric acid)	Fisher Scientific
HEPES	Sigma-Aldrich
Fas ELISA kit	R&D Systems
Caspase-3 ELISA kit	R&D Systems
Hybride nitrocellulose membrane	Amersham Bioscience
Isopropanol	Sigma-Aldrich
Kanamycin	Sigma-Aldrich
Magnisium chloride	Sigma-Aldrich
Methanol	Fisher Scientific
Methotrexate	Sigma-Aldrich
Milk powder	Marvel Original, Dublin
Na_2HPO_4	BDH Lab supplies, England
NHS (Normal Human Serum)	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
PBS (Phosphate Buffer Saline)	Fisher Scientific
Penicillin/Streptomycin	Fisher Scientific

Phalloidin	Invitrogen
Ponceau S	Sigma-Aldrich
Potassium chloride	Sigma-Aldrich
Propidium iodide	Sigma-Aldrich
Propylene oxide	Agar Scientifics
Protein molecular weight marker	BioRad
PSG (Phosphate solution+ glucose)	CMIRC
Reynolds Lead Citrate stain	Agar Scientifics
RPMI	Fisher Scientific
SB-431542	Sigma-Aldrich
SDS (Sodium dodecyl sulphate)	Sigma-Aldrich
Sodium azide	Avocado Research Chemicals
Sodium chloride	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
TEMED	Fisher Scientific
Tris Base	Sigma-Aldrich
Trichloroacetic acid	Fisher Scientific
Triton X-100	Sigma-Aldrich
Trypsin/EDTA solution	Sigma-Aldrich
Tween 20	Sigma-Aldrich

2. Technical Equipments

AMT Digital camera	Advanced Microscopy Tech
Bacterial incubator	Heraeus Incubator
Cell cultur flasks ($75cm^2$)	Fisher Scientific
Centrifuge 5804 R	Eppendorf
Centrifuge 5810 R	Eppendorf
Gel loading tips	Corning

Guava EasyCyte Flow cytometer	Guava Technologies, UK
Fluorescence microscope (1X81)	Olympus Corporation, Germany
FLUOstar Omega plate reader	BMG Labtech, UK
Incubator Herraeus CO_2-Auto-Zero	Thermo Electron Corporation
Joel JEM-1200Electron microscope	Debden UK Ltd
Leica Ultracut R ultra microtome	Leica, Wein, Austria
Microcentrifuge 5417R	Eppendorf
Microplate (12-well)	Sigma-Aldrich
Microplate (24-well)	Sigma-Aldrich
Microplate (96-well)	Sigma-Aldrich
Nikon Inverted microscope, TS 100	Nikon Eclipse, Japan
pH-Meter 766 Calimatic	Jenway
Pioloform film copper grids	Agar Scientifics
Roto-Shake Genine	Denley
Semi-dry transfer system	BioRad
Small volume tips	Sigma-Aldrich
Sorvell ultracentrifuge RC6	Thermo Electron Corporation
Sorvell T-865 rotor	Sorvell
F-20 micron rotor	Sorvell
SE-12 rotor	Sorvell
Sonicating waterbath	Townson & Mercer Ltd, Croydon
Spectrofluorometer	Lambda Advanced Technology
UVP Chemiluminescence	UVP Bioimaging Systems, UK

3. Antibodies

Anti-Annexin V Alexa Fluor 488	eBiosciences
Mouse anti-CVB1	Chemikon
Mouse anti-fas	Millipore
Caspase3-inhibitor	R&D Systems
Mouse anti-fas ligand	Millipore
Mouse anti-IgG FITC	AbD Serotec
Anti-LAMP-1 Alexa Fluor 488	eBiosciences
Mouse anti-actin	Kind gift
Mouse anti-CAPNS1	Sigma-Aldrich
Rabbite anti-caspas-3	abcam