Cellular Communication via Microparticles - Role in Transfer of Multidrug Resistance in Cancer.

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Abbreviations: Microparticles, MPs; Multidrug Resistance, MDR; Multidrug Resistance-Associated Protein 1, MRP1; micro RNA, miRNA; P-glycoprotein, P-gp.

ABSTRACT
Multi-drug resistance (MDR) continues to be a major impediment in the successful treatment of cancer. The two efflux transporters, P-glycoprotein (P-gp) and the MDR-Associated Protein (MRP1) are major contributors to cancer MDR clinically. The upregulation of P-gp leading to MDR was initially understood to occur via pre- and post-transcriptional mechanisms only. However, we demonstrated that microparticles (MPs) mediate the intercellular exchange and trafficking of bioactive material including functional P-gp and selected modulatory miRNAs. This exchange of P-gp leads to the dissemination of MDR within a cancer cell population. These findings have significant implications in understanding the cellular basis governing the intercellular acquisition of deleterious traits in cancers, serving to substantially advance our understanding of the molecular basis for the emergence of MDR in cancer clinically.

Keywords: Cancer, CD44, Cellular communication, Ezrin, Microparticle, microRNA, Multi-drug Resistance, Multi-drug Resistant Associated Protein 1, P-glycoprotein, Trait dominance.

The clinical problem of drug resistance in the treatment of cancer

Although targeted therapies provide a rational and selective modality in cancer therapeutics, the development of drug resistance and the subsequent result of tumour unresponsiveness continue to plague clinical oncology. Drug resistance is seen when tumours that have been responding favourably to chemotherapeutic treatment suddenly reoccur or when the tumour fails to respond to initial treatment [1]. Numerous mechanisms contributing to drug resistance have been reported. These include reduced drug uptake, mutated and/or altered expression of drug targets, enzymatic inactivation of drugs, drug efflux mechanisms and alterations in apoptosis, senescence and repair mechanisms. In addition to resistance to a single drug, cancer cells often display a cross resistance to a diverse range of unrelated drugs resulting in a phenomenon known as multidrug resistance (MDR).

Multi-drug resistance in cancer

Multi-drug resistance (MDR) is typically characterized by a cross resistance to a wide range of pharmacologically unrelated drugs following the exposure of the cancer to a single anticancer agent [2]. MDR is mainly attributed to a reduced intracellular drug accumulation in cancer cells by virtue of increased drug efflux from within the cancer cell [3]. Consequently, sub-lethal intracellular drug concentrations are maintained, and the cell survives cytotoxic drug exposure. Classical MDR is frequently attributed to the elevated expression of members of the ATP Binding Cassette (ABC) superfamily of membrane transporters. P-glycoprotein (*ABCB1/*MDR1/P-gp) and the MDR Associated Protein 1 (*ABCC1/*MRP1) comprise two of the most widely studied molecules that underpin mechanisms of active efflux implicated in cancer MDR.

P-gp and MRP1 and their role in conferring MDR

P-glycoprotein (P-gp/MDR1/ABCB1):P-gp is a 170 kDa phosphoglycoprotein, encoded by the human *ABCB1* gene, located on the long arm of chromosome 7, which utilizes energy released from ATP hydrolysis for drug efflux across cell membranes [4]. P-gp is a promiscuous drug transporter with a physiological role in binding and effluxing a wide array of structurally and functionally unrelated compounds thereby protecting both cells and the organism as a whole from xenobiotics. This remarkable efflux capacity reduces the intracellular concentration of a wide range of chemotherapeutics, leading to MDR and anticancer treatment failure. Most, but not all P-gp substrates are hydrophobic organic compounds of large molecular weight (> 400), that are amphipathic, possess a planar ring system and carry a positive charge at physiological pH [5]. P-gp substrates therefore span various therapeutic drug classes including; typical anticancer agents (anthracyclines, vinca alkoloids, taxanes), HIV protease inhibitors, antipsychotics, natural products (colchicine and curcuminoids), linear and cyclic peptides, steroids, fluorescent dyes, γ-emitting radiopharmaceuticals and many other miscellaneous agents [2, 6].

The expression of *MDR1* or *ABCB1* gene is often related to poor remission and survival rates across many malignancies, serving as a predictive indicator of anticancer treatment failure. P-gp overexpression is observed across many human cancers including hematopoietic cancers such as acute myeloid leukaemia [7, 8] and acute lymphoblastic leukaemia [9]; childhood cancers such

as soft tissue sarcoma [10], neuroblastoma [11] and fibrosarcoma [12]; gastrointestinal tract and genitourinary system malignancies [12], osteosarcoma [13], breast cancer [14], non-small cell lung carcinoma [15] and prostate cancer [16]. P-gp related MDR may be classified as "intrinsic" in tumours of the colon, renal cell carcinomas, hepatocellular carcinomas, pancreatic carcinomas and pheochromocytoma, which innately express high P-gp [17-19]. These tumours usually fail to respond to primary treatment, by virtue of their intrinsic resistance to therapy. On the other hand, in many other malignancies, P-gp overexpression is induced during therapy whereby initially responding tumour returns refractory to many agents contributing to "acquired" MDR [2]. Such malignancies include that of the breast and the ovary [20, 21].

MDR associated protein 1 (MRP1/ABCC1):MRP1, encoded by the human *ABCC1* gene (located on chromosome 16) [22], similarly has an extraordinary capacity to transport a wide range of substrates. Although a significant overlap exists with the substrate specificity of P-gp, MRP1 also transports diverse physiological substrates including; glutathione [23], glucuronide and sulphate conjugates, organic anions and heavy metals [24, 25]. Many of its substrates are xenobiotics used as antineoplastic agents including the folate-based antimetabolites, anthracyclines and anti-androgens [25]. The clinical relevance of MRP1 overexpression has been associated with several human malignancies such as a wide range of haematological and solid tumours [26]. Its overexpression is implicated in MDR, being correlated with poor prognosis and survival in non-small cell lung carcinoma [27], breast cancer [28], prostate cancer [16] and childhood neuroblastoma [29].

a. P-gp and MRP1 structural homology. P-gp (170 kDa) and MRP1 (190 kDa) are single polypeptides, topologically composed of a minimum of four core functional elements; namely two transmembrane domains (TMD_1 andTMD_2) forming the pathway for transport of substrates and two nucleotide binding domains (NBD_1 and NBD_2) that hydrolyse ATP for the efflux function [30]. Each TMD in P-gp typically consists of six putative α-helices. The topology of MRP1 is similar to that of P-gp, but it possesses an additional N-terminal domain with five putative α-helical TMD (TMD_0) of unknown function [2, 31].

b. Transporter functional redundancy. The human genome encodes for 49 ABC transporters of which, those with multidrug resistance capabilities are ubiquitously expressed at physiological barriers in the body. Specifically, P-gp is highly expressed on the apical membranes of the intestine, colon, pancreas and renal proximal tubule, hepatocytes and in the endothelial cells lining capillaries in the brain, testis, placenta and inner ear [6]. Likewise, although MRP1 is ubiquitously expressed in the body, highest expression levels are found in the lung, testis, kidney, heart and placenta [27]. Consistent with their localisation at pharmacological barriers, these transporters serve an essential role in protecting both vital organs and the organism as a whole from xenobiotic exposure. A functional redundancy exists among these transporters, which may be attributed to their significant tissue colocalisation, broad and overlapping substrate specificities and a significant sequence homology. This redundancy ensures a fail-proof survival mechanism for preserving the organism from xenobiotic insult. Similar to P-gp, MRP1 has been reported to be transferred via MPs and is functional in a time dependent manner in

drug sensitive leukaemia cells. Following their MP-mediated transfer, a kinetic difference between P-gp and MRP1 becoming functional in the recipient cells has been reported [32]. Another transporter *ABCG2* plays a role in MDR, however nothing has been reported on their role in MP-mediated transfer. This is currently under investigation in our lab.

Almost half the members (namely *ABCA1-12*, *ABCB1-11, ABCC1-12, ABCG)* of this family have a role in conferring drug resistance *in vitro* and clinically [31] and have various immune system roles [34, 35] evading tumour immune responses [36], making them ideal therapeutic targets. However, the existing functional redundancy has complicated attempts for the pharmacological circumvention of P-gp mediated MDR. Current drug discovery screening programs include the development of MDR reversal agents that can simultaneously inhibit multiple transporters rather than single entities [6]. However, this approach is clinically limited, due to the poor specificity, low affinity for the binding site and interference with the physiological role of these transporters expressed at pharmacological barriers [37], resulting in severe side effects.

c. Cytoskeletal anchorage of P-gp to the cell membrane. Interactions between the plasma membrane and the cytoskeleton are essential in cell adhesion, cell signalling, membrane trafficking, cell motility, apoptosis and MP formation [38-40]. The FERM domain (F for 4.1 protein, ezrin, radixin and moesin) proteins play an important role in these processes and possess a unique module required to interface membrane proteins with the cytoskeleton. These specialized groups of macromolecules are found in higher order structures including sites of cell-cell and cell-to-extracellular matrix attachment [41]. Furthermore, FERM domain proteins have been shown to be responsible for the direct association between MDR proteins and the cytoskeleton. Specifically, P-gp colocalises with ezrin in IFN-γ treated monocyte derived macrophages [42] and with ezrin, radixin and moesin (ERM) proteins on pseudopods/ uropods in resistant lymphoid cells [38]. In addition, there could be a potential indirect involvement of lipid micro-domains such as caveolae in the cytoskeletal anchorage of P-gp to the cell membrane. Indeed, P-gp and MRP1 have been shown to be localised in caveolae and the expression of Caveolin-1 may directly modulate the functional activity of P-gp [43-44]. Disruption of the ERM–P-gp association impairs P-gp function and results in a cellular redistribution of P-gp [38], supporting an essential role for the ERM proteins in the plasma membrane localisation of P-gp. Consistent with this, the actin-P-gp interaction is also required for the endosomal trafficking of P-gp to the plasma membrane [38, 45]. These studies establish that the membrane localisation of P-gp occurs by direct actin anchorage through the FERM domain proteins [46]. We recently showed that ezrin was present in both the parental leukaemia and breast cancer cells and their P-gp over expressing MDR variants. In addition, the levels of ezrin was also found to be dependent on the MDR protein levels [47].

Another cell adhesion molecule that interacts with P-gp via FERM domain binding proteins is CD44. CD44 is the major surface receptor to hyaluronan, which is involved in cell adhesion, metastasis and motility [48]. CD44 assembles intracellular complexes involving the FERM domain proteins, the binding of which, anchors CD44 to actin and influences downstream signalling. CD44 and P-gp interact in carcinoma cell lines to promote cell migration and invasion

[49]. Both CD44 and P-gp can be co-immunoprecipitated, are colocalised and their expression coregulated, supporting a molecular interaction between the two proteins [49, 50]. P-gp complexes with activated CD44 via ERM intermediate with membrane co-localisation dependent on CD44 interaction [51].

Non-genetic modulation of MDR acquisition

Modulation of MDR expression was understood to exclusively occur endogenously via pre- or post-transcriptional mechanisms. Increased mRNA as a result of gene amplification, enhanced gene transcription and increased mRNA half-life [52, 53], gene amplification and increased mRNA stability due to prolonged exposure to several cytotoxic drugs [54] have been shown to result in MDR overexpression in cancer cells. In addition, modulation in protein stability, plasma membrane incorporation [55] and increased P-gp trafficking [21] have been reported for P-gp upregulation at the protein level. Although the genetic basis for MDR acquisition is well characterized, little was known about the role of non-genetic mechanisms in the overall acquisition of this phenotype (Figure 1A). The non-genetic acquisition of membrane proteins via cell-cell communication modalities provides an efficient and alternative pathway for the cellular acquisition and dissemination of traits. Various supramolecular mechanisms involving direct cell-cell contact [56], cellular membrane blebs like microparticles (MPs) [57], exchange of membrane fragments or trogocytosis [58], tunnelling nanotubes [59, 60] and cytoneme or filopodial bridges [61] have been observed to form the basis of cellular communication leading to intercellular membrane protein transfer. Some of these mechanisms are depicted in Figure 1B.

Microparticles play a unique role in intercellular communication

Among the most intriguing mechanisms of intercellular exchange is the generation and intercellular trafficking of MPs. MPs serve as natural physiological vehicles, via which cell surface and intracellular molecules exit the donor cell, are carried to another cell (long and short range) in the intact state and exert their effect on recipient cell populations. Levchenko and coworkers demonstrated the intercellular transfer and 'non-genetic' acquisition of P-gp in MDR [56]. The intercellular transfer of functional P-gp from P-gp (+) donor cells to P-gp (-) recipient cells was demonstrated following co-culture of parental cells with their MDR derivative cells. On the basis of filtered medium experiments, the authors concluded that protein transfer was mediated by direct cell to cell contact. The non genetically acquired phenotype was unstable and required a constant exposure to either a selecting pressure (an anticancer drug) or the presence of MDR cells so as to maintain the acquired phenotype [56]. Mack and co-workers, in 2000 demonstrated the role of MPs in transferring CCR5, a 62 kDa chemokine receptor. They showed that CCR5, the principal co-receptor which enables the transmission and propagation of macrophage-tropic human immunodeficiency virus (HIV-1), were transferred by microparticels from the surface of CCR5 (+) cell to recipient CCR5 (-) cells, conferring susceptibility to HIV-1 infection [62]. In 2009, we demonstrated that MPs serve as intercellular vectors of MDR dissemination, and acquisition via intercellular transfer of functional P-gp [63]. Since our initial finding we have shown MPs to play an even more alarming role in cancer biology by; (i) incorporating and transferring a variety of MDR proteins (both P-gp and MRP1) and nucleic acids [32, 64, 65], (ii) re-templating the transcriptional landscape of recipient cells to ensure the acquisition of deleterious cancer traits [32, 65], (iii) displaying MDR proteins in inside out orientation in MP exposed recipient cells, with respect to untreated cells across different

malignancies (Gong *et al.,* 2013, EJP, in press) and (iv) by sequestering anticancer drugs within their intravesicular space, thereby reducing the amount of free drug available to cancer cells (Gong *et al.,* 2013, EJP, in press). This serves as a parallel pathway in the acquisition of MDR in malignancies.

a. Microparticles in physiology and pathology. MPs are small membrane vesicles (0.1-1 μm in diameter) derived from the ubiquitous cellular phenomenon of plasma membrane budding [66]. MPs display phosphatidylserine (PS) on their surface and are distinguished from exosomes by size (exosomes typically ranging 40-100 nm in diameter), phenotype and origin (exosomes originate from endocytic bodies) [67]. MPs are released, under normal physiological conditions, from the plasma membranes of various cell types including; platelets, macrophages, monocytes, T-cells, endothelial cells and erythrocytes [68-70]. However, in response to cell stimulation or stress, the vesiculation undergoes a dramatic acceleration and qualitative change [71].

MPs were initially considered as inert side products of cellular activation. They have now been reported as important intermediaries in inflammation, coagulation and vascular homeostasis [72]. Many pathological conditions and diseased states such as autoimmune disorders, atherosclerosis, HIV infections, cerebral malaria, sepsis [73-76] and cancer [63, 64] have been reported to have elevated levels of systemic MPs. In addition, MPs shed from cancer cells are associated with tumour cell invasiveness [77], evasion of immune surveillance [78], angiogenesis [79], chemoresistance [63, 65] and contribute to the hypercoagulable state observed in many malignancies [80,81], thereby acting as important mediators in paraneoplastic syndromes (Figure 2).

***b. Microparticle biogenesis*.** MPs are released upon cellular activation or during apoptosis following a breakdown of the plasma membrane's natural phospholipid asymmetry and a remodelling of the cytoskeleton [2, 66]. At steady state, the cell membrane displays an asymmetric phospholipid configuration. Upon cell activation, an increase in intracellular calcium modulates the enzymatic regulators governing phospholipid asymmetry, resulting in a scrambling of lipids between the two membrane leaflets. The increase in cytosolic calcium activates the enzyme, calpain which serves to hydrolyse the actin binding proteins and disrupt the cytoskeletal scaffold immediately under the membrane bilayer. The structural loss facilitates membrane budding and MP shedding from the cell [82].

c. Microparticle cargo. Upon release, MPs carry cellular proteins, second messengers, growth factors and genetic material from their cells of origin [2, 83] and comprise the major source of RNA (ribosomal RNA (rRNA), messenger RNA, (mRNA) and microRNA (miRNA) in systemic circulation [2, 64, 84-86]. MPs also carry the transcripts of enzymes responsible for its biogenesis (floppase and scramblase) together with the transcripts of enzymes required for miRNA biogenesis (Dicer, Drosha and Argonaute) [64]. The intravesicular localisation of nucleic acid cargo in the MPs prevents their systemic degradation by blood nucleases, thereby constituting MPs as major source of RNA in systemic circulation [86]. Given that MPs are emerging as an important source of miRNA in the circulation of cancer patients [87, 88] it is conceivable to propose a role for MP in the aberrant miRNA levels displayed in oncogenesis and, potentially, in metastasis. Indeed the detection of circulating tumour-derived transcripts from

melanoma, breast and lung cancer patients have defined MPs as markers of diagnostic and prognostic significance [64, 87].

In addition, MPs represent a concentrated source of bioactive molecules and have been shown to relay significant detrimental effects following their transfer onto target cells *in vitro*. A process of selective packaging or sorting of cargo is operational in the MPs, whereby MPs harbour differential amounts of the bioactive material with respect to their donor cells [64, 65]. Upon transfer, we have shown MPs can impose the dominant donor cell trait onto recipient cells, thereby effectively *re-templating* the transcriptional landscape of recipient cells to ensure the acquisition of deleterious cancer traits [64, 65].

We recently showed that MPs cargo also includes cytoskeletal anchorage proteins such as ezrin and CD44, which are associated with membrane localisation of P-gp. Briefly, we demonstrated that the ezrin was present in both the parental and their P-gp over expressing MDR variants of leukaemia and breast cancer cells. MPs derived from the drug resistant cells selectively packaged higher levels of ezrin, with respect to their donor cells. Upon co-culture of these drug resistant MPs with parental recipient cells, we did not observe any significant increase in ezrin over and above the endogenous levels already present in the recipient cells [47].In addition to ezrin, we have shown that breast cancer-derived MPs selectively package CD44 (isoform 10) with respect to its comparator the leukaemia-derived MPs [47]. Furthermore, we demonstrated that the breast-cancer derived MPs display tissue selectivity in transferring P-gp to malignant cells only in comparison to the leukaemia derived MPs, which effectively cross-talk across both malignant and non-malignant cells [47]. We proposed that the differential presence of CD44 (isoform 10) on the breast cancer derived MPs may contribute to the observed P-gp transfer selectivity [47].

microRNA cargo. miRNAs are highly conserved, single-stranded non-coding regulatory RNA, typically 19–25 nucleotides in length. These nucleic acids modulate the activity of specific mRNA targets by pairing with partial complementary sites in the 3'- untranslated region (UTR) of target genes [2]. miRNA synthesis begins in the nucleus by RNA polymerase II to form primary miRNA (pri-miRNA). Pri-miRNA is processed by the ribonucleases, *Drosha* and *Dicer* to generate mature miRNA. The single stranded miRNA, in association with *Argonaute 2*, binds to complementary sequences in the 3' untranslated region (UTR) of target transcripts to regulate gene expression either by translational repression, activation or degradation of the mRNA transcript [2].

At present over 1000 human miRNAs have been identified targeting an estimated 30% of human genes [89], serving as important regulators of a wide range of pathophysiological processes [90]. By targeting several genes, miRNAs play important roles in complex pathophysiological networks including cell proliferation, differentiation, apoptotic cell death, stress resistance, physiological metabolism and resistance to chemotherapeutic agents [91-94]. Consequently, aberrant expression of miRNAs has been associated with malignancy, including; cancer stage, disease progression and metastatic spread [64, 95, 96]. Specifically, we have demonstrated that

certain miRNAs are selectively packaged in MPs derived from both haematological and non-haematological cancer cells. These miRNAs are involved in pathways implicated in cancer pathogenesis, membrane vesiculation and cascades regulated by ABC transporters [64]. MPs incorporate miRNAs and act as vectors, facilitating their intercellular functionality between cells [64, 97, 98]. We also showed that following MP co-culture with recipient parental cells, miRNA expression trends of the MP acquired recipient cells were reflective of that of the MP donor cells. Thereby, demonstrating that the recipient cells reflect the donor trait following MP-mediated transfer of cargo [64].

miRNA and MDR. miRNAs play an important role in the regulation of chemoresistance. Resistance to topetecan, doxorubicin, methotrexate, docetaxel and cisplatin have been correlated with alterations in miRNA expression in tumour cells [99-104]. However, very little was known about the role of miRNAs in ABC transporter expression and function. *miR-27a* and *miR-451* expression were shown to activate MDR1/P-gp expression in resistant human ovarian cancer [65, 105]. We demonstrated the involvement of *miR-27a* together with the modest amount of transcript delivered by MPs to contribute to a modest increase in *ABCB1* levels in breast cancer cells [65]. Consistent with earlier observations in breast cancer tissues [106], we identified an inverse relationship between MRP1 mRNA and *miR-326* levels in leukaemia cells [65]. Likewise, the overexpression of *miR-21* and the downregulation of the tumour suppressor protein PDCD4 in breast cancer has been shown to upregulate P-gp expression leading to chemoresistance [107]. *miR-345* and *miR-7* have been shown to target MRP1, with the former displaying lower expression in MDR breast cancer cells relative to parental cells [103]. Recently, *miR-297* was shown to play a role in the development of MDR by the modulation of MRP2 in colorectal cancer cells [108]. These emerging data substantiate a role for miRNAs, including that conferred onto recipient cells by MPs in contributing to the emergence of MDR and regulation of transporter expression in cancer cells.

Role of microparticles in conferring P-gp mediated MDR in Cancer

MPs carry surface antigens, cytoplasmic and nuclear constituents from their originating cell [62, 64, 109] and mediate intercellular cross-talk by transferring receptors, antigens and cytokines from donor cells to recipient cells [66]. The presence of cell adhesion molecules on MP supports a capacity for target cell binding and intercellular crosstalk [62]. We first described a novel "*non-genetic*" mechanism for the acquisition of MDR, whereby MPs serve as vectors in the intercellular transfer of functional P-gp from MDR donor cells to drug sensitive recipient cells (Figure 1A) [63]. To our knowledge, this was the first report that a protein as large as P-gp, a 170 kDa polypeptide, consisting of 1280 amino acids that spans the plasma membrane 12 times [110], could be transferred by submicron membrane vesicles, into recipient cells whilst retaining its functional state. Furthermore this intercellular pathway occurs across haematological and non-haematological malignancies [65] with P-gp transfer occurring as early as 2 hours and functional MDR acquired within 4 hours of transfer [63]. However, it still remains to be elucidated as to how MPs are incorporated within the recipient target cells enabling the effective transfer of functional P-gp and other antigens across cells. Although the exact mechanism is still unclear, previous studies have shown that on interaction with recipient human brain endothelial cells, platelet derived MPs are internalised within vesicular structures and their different components are either degraded or recycled/endocytosed inside the target

cell [111]. This is currently under study and we suggest that processes such as MP membrane fusion, endocytosis, phagocytosis may be involved.

Since these initial findings we have demonstrated using direct immunolabelling and flow cytometric analysis, that the extent of MP-mediated transfer of P-gp is dependent on MP amount (Figure 3). This data shows that as little as 30 μg of total MP protein is able to transfer functional P-gp (10% total P-gp with respect to parental cells) to recipient drug sensitive cells. In addition, 180 μg of total MP protein is observed to be optimal for maximal P-gp transfer and function (lowest intercellular drug accumulation) (Figure 3). Several reports have demonstrated that elevated numbers of platelet-derived MPs are present in metastatic gastric [112], breast [113, 114] and pancreatic cancer [113]. In addition, a recent study showed that plasma samples of breast cancer patients had 10,000 $x10^6$/L number of total MPs higher than the control samples [115]. Extrapolating this MP number to our study, as little as $3x10^5$ number of MPs (30 μg) as used in *in vitro* is clinically relevant under physiological conditions. We further validated the transfer of functional P-gp following whole cell drug exclusion assays using two distinct fluorescent P-gp drug substrates, daunorubicin (DNR) and rhodamine 123 (Rh123) (Figure 4). These studies confirm that MP-mediated transfer of P-gp results in the dissemination of the MDR phenotype whereby cells exhibit cross-resistance to unrelated substrates such as DNR and Rh123, *in vitro*, consistent with our previous reports [63].

Stability of the acquired trait

We have shown that MPs mediate the transfer of MDR proteins from donor cells to drug sensitive cells, thereby conferring the MDR phenotype onto the recipient cell. In addition, we demonstrate that the acquired MDR trait in the recipient cell is stable for at least 5 days *in vitro*, in the absence of any selecting pressure such as cytotoxic drugs or subsequent exposure of MPs themselves (Figure 5).

Our *in vivo* studies conducted using a murine MCF-7 tumour xenograft model show that MP-mediated P-gp is rapidly acquired by drug sensitive tumours within 24 hours of MP exposure with P-gp localising deep within the tumour core. This acquired phenotype is stable for at least 2 weeks in the absence of further MP exposure or a selective pressure (drugs or resistant cells) [47]. The stability and the occurrence of this pathway *in vivo* further emphasize the severity and the deleterious effect of MPs in conferring MDR and other deleterious traits in cancer, with potential clinical significance.

Implications for alternative treatment strategies and future perspective

Over the past decade MP biology has emerged quickly and has been attracting growing interest in the context of disease pathophysiology. MPs are mostly identified as important natural vehicles in which intracellular macromolecules are packaged and exported to another cell, either locally or distant from their site of origin, in the intact state. Although very little is currently

known about the clearance of MPs, it has been suggested that the interactions of MPs with other cells may represent a mechanism for their elimination from the circulation [73]. Indeed, there has been a recent report on the clearance of transfused platelet derived MPs (PMP) in human blood, with a half-life-time of ~6 hours [116].

MDR is major obstacle to effective chemotherapy. In addition to this, the coexistence of a 'non-genetic' mechanism of dissemination complicates the situation further. At present, the area of MP-mediated transfer of MDR is an emerging field with no clinical study available to date. Studies done till date have generally used highly drug resistant cell lines expressing very high levels of P-gp. Clinical implications of MP-mediated MDR will be supported by further future research in patient tumour samples expressing physiologically low levels of P-gp. However, our studies in both leukaemia and breast cancer have shown MPs to be important mediators in the intercellular transfer of MDR and other deleterious cancer traits *in vitro* and *in vivo*. The elucidation and further exploration of this novel pathway could provide a mechanistic understanding of the intercellular acquisition of deleterious traits in cancer clinically. The demonstration of the role of MPs in the transfer of P-gp and acquisition of MDR has important implications for developing therapeutic strategies to prevent the spread of MDR clinically. Thus it is inferred that in addition to the current MDR treatment strategies that incorporate conventional P-gp inhibitors, once clinically proven, it would be worthwhile to consider inhibition of the MP-mediated component of this pathway [117]. MP production has been linked to cancer progression and metastasis; hence inhibiting MP formation (by several known MP inhibitors such as calpain inhibitors, calcium channel blockers, ROCK inhibitors and pantethiene) [117] may have the potential to not only hinder cancer cell proliferation but also their ability to transfer MDR. In addition, MPs from biological fluids could provide a non-invasive diagnostic predictive biomarker, through their nucleic acid and protein signatures in certain disease states. Dissecting the nature and implications of this pathway represents a new exciting challenge in cancer biology and clinically in cancer therapeutics.

EXECUTIVE SUMMARY

The clinical problem of drug resistance in the treatment of cancer

- Tumour unresponsiveness to chemotherapy is a major concern in cancer treatment.
- Drug resistance is a multimodal phenomenon.
- Multi-drug resistance (MDR) is more serious still where cancer cells display cross-resistance to diverse drugs.

Multi-drug Resistance in cancer

- MDR is characterised by reduced intracellular drug accumulation attributed to the overexpression of two drug transporters, P-gp and MRP1 in cancer cells.
- A functional redundancy exists among these transporters, ensuring a fail-proof survival mechanism for the organism, but complicating pharmacological circumvention of P-gp mediated MDR.
- FERM domain proteins and CD44 associate with P-gp leading to the interaction with the plasma membrane and cytoskeleton, essential in cell adhesion, metastasis and motility.

- MPs derived from the drug resistant cells selectively package higher levels of ezrin, with respect to their donor cells.
- CD44 (isoform 10) is present on the breast cancer derived MPs but not leukaemia derived MPs, which may play a role in P-gp transfer selectivity displayed by these MPs.

Non-genetic modulation of MDR acquisition

- Several cell-cell communication modalities (direct cell-cell contact, microvesicles and microparticles, trogocytosis, tunnelling nanotubes and cytoneme or filopodial bridges)provide an alternative non-genetic pathway for the cellular acquisition and dissemination of MDR traits.

Microparticlesplay a unique role in intercellular communication

- MPs are important clinical mediators of various pathophysiological processes including inflammation, coagulation, vascular homeostasis, HIV-1 and cancer.

- MPs serve as intercellular vectors of MDR dissemination, and acquisition via intercellular transfer of functional P-gp.

- MP cargo includes cellular proteins, second messengers, growth factors, genetic material (DNA, RNA, miRNA), transcripts of enzymes responsible for MP biogenesis and miRNA biogenesis, ezrin and CD44 from their cells of origin.

Role of microparticles in conferring P-gp mediated MDR in Cancer

- MP mediated transfer of P-gp occur as early as 2 hours and functional resistance acquired within 4 hours.

- MP-mediated transfer of P-gp is dependent on MP amount.

Stability of the acquired trait

- The acquired MDR trait in the recipient cell is stable for at least 5 days *in vitro*, in the absence of any selecting pressure or subsequent exposure to MPs themselves.

- In *in vivo* studies, MP-mediated P-gp is rapidly acquired by drug sensitive tumours within 24 hours of MP exposure and this acquired phenotype is stable for at least 2 weeks in the absence of further MP exposure or a selective pressure.

Implications for alternative treatment strategies and future perspective

- The elucidation and further exploration of MP-mediated transfer of MDR provides a mechanistic understanding of the intercellular acquisition of deleterious traits in cancer clinically.

- This pathway has important implications for developing novel therapeutic strategies to prevent the spread of MDR.

- MPs from biological fluids could provide a non-invasive diagnostic predictive biomarker.

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Figures

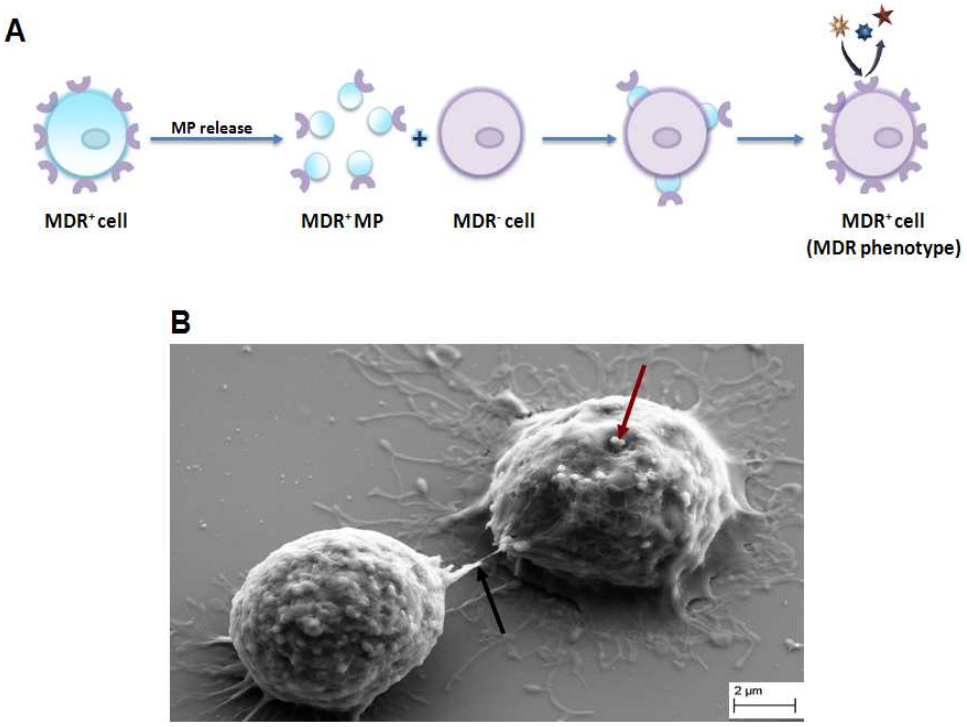

Figure 1: (A) Mechanism of MP-mediated MDR. P-gp may be acquired by non-genetic mechanisms, including MP mediated MDR where MPs carrying P-gp from their originating cells are spontaneously shed from drug MDR$^+$ donor cells. Shed MPs bind to drug-sensitive recipient cells and transfer functional P-gp to confer the MDR phenotype. **(B) Scanning Electron Microscopy Image depicting the modes of intercellular communication:** Human acute lymphoblastic leukaemia (CCRF-CEM) cells facilitate intercellular communication via tunnelling nanotubes (black arrow) and also by microparticles amongst others (red arrow).. The cells were imaged using the **Zeiss ULTRA** *plus*scanning electron microscope following fixation with osmium tetroxide and coating with platinum. Scale bar as shown in panel.

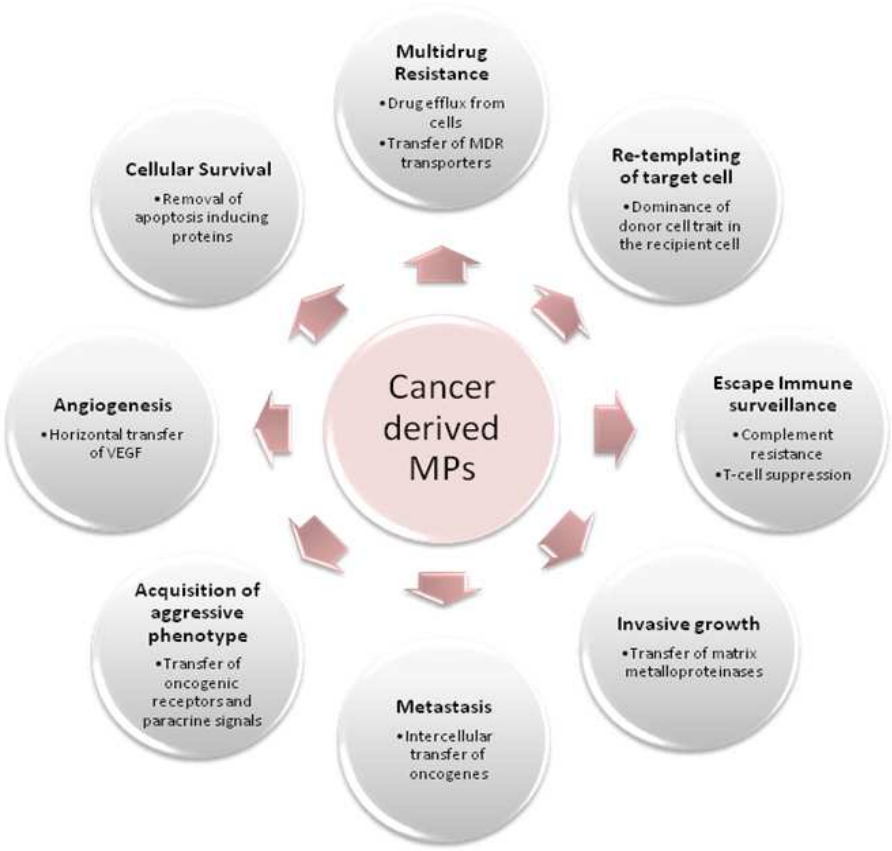

Figure 2: Role of cancer derived MPs in cancer biology: Tumour–derived MPs affect various aspects of cancer biology, through their ability to selectively carry bioactive molecules and also to act as vectors for the horizontal transfer of their cargo.

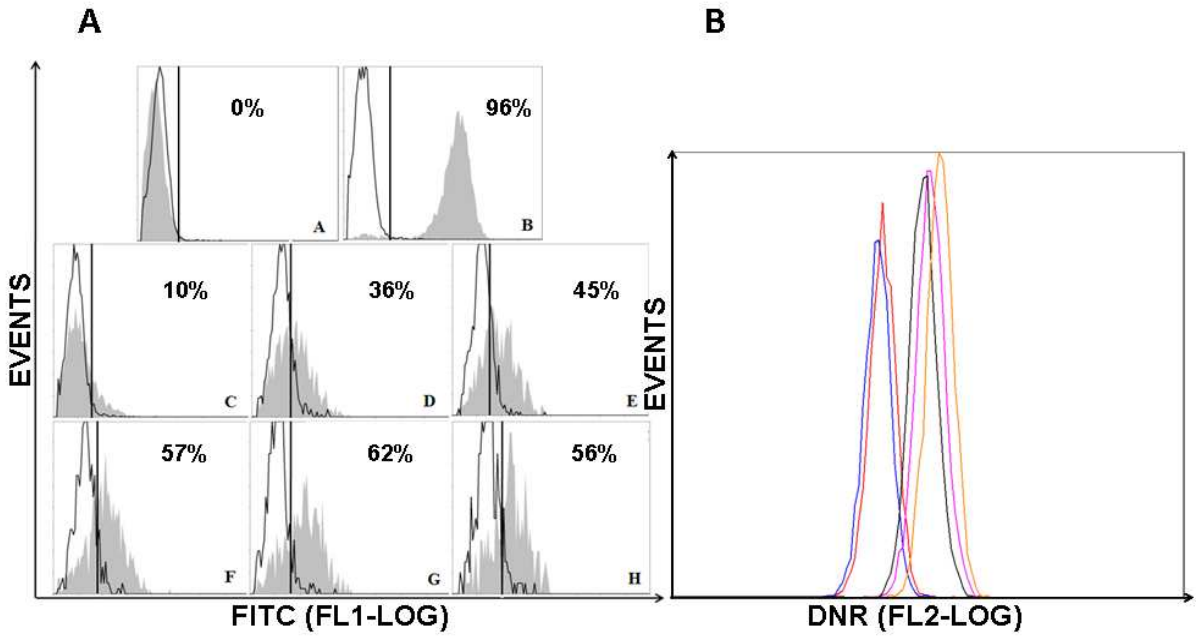

Figure 3: MP transfer of P-gp from MDR$^+$ donor drug resistant cells to drug sensitive (MDR$^-$) recipient cells. (A) Surface P-gp expression following MDR$^-$ cell co-culture with MDR$^+$MP. Cells were labeled with FITC-anti-P-gp (solid) or isotypecontrol (open) mAbs and analyzed by flow cytometry. Histograms depict percent P-gp positive populations, **A**: MDR$^-$, **B**: MDR$^+$, **C:** MDR$^-$+MP 30 µg, 10% of MP exposed recipient cells expressed P-gp compared to untreated cells. **D:** MDR$^-$ +MP 80 µg, 36% of MP exposed recipient cells expressed P-gp compared to untreated cells. **E:** MDR$^-$+MP 100 µg, 45% of MP exposed recipient cells expressed P-gp compared to untreated cells. **F:** MDR$^-$+MP 150 µg, 57% of MP exposed recipient cells expressed P-gp compared to untreated cells. **G:** MDR$^-$+MP 180 µg, 62% of MP exposed recipient cells expressed P-gp compared to untreated cells and **H:** MDR$^-$+MP 250 µg, 56% of MP exposed recipient cells expressed P-gp compared to untreated cells..**(B) MP transfer of functional P-gp.** Mean fluorescence intensity (MFI) of daunorubicin (DNR) accumulation in MDR$^-$ cells with exposure to increasing amounts of MPs. The recipient cells post 4 h co-culture were treated with 1µM DNR for 1hr at 37^{0}C and intracellular drug accumulation detected using flow cytometry. DNR accumulation in —: MDR$^-$(MFI-14.5),—: MDR$^-$+MP 15 µg (MFI-10.7), —:MDR$^-$+MP 40 µg (MFI-9.31), —: MDR$^-$+MP 160 µg (MFI-4.45) and —:MDR$^-$+MP 180 µg (MFI-4.11). 180 µg MP in co-culture results in maximal P-gp transfer (A) and function (lowest intracellular DNR accumulation) (B). Data are representative of a typical experiment.

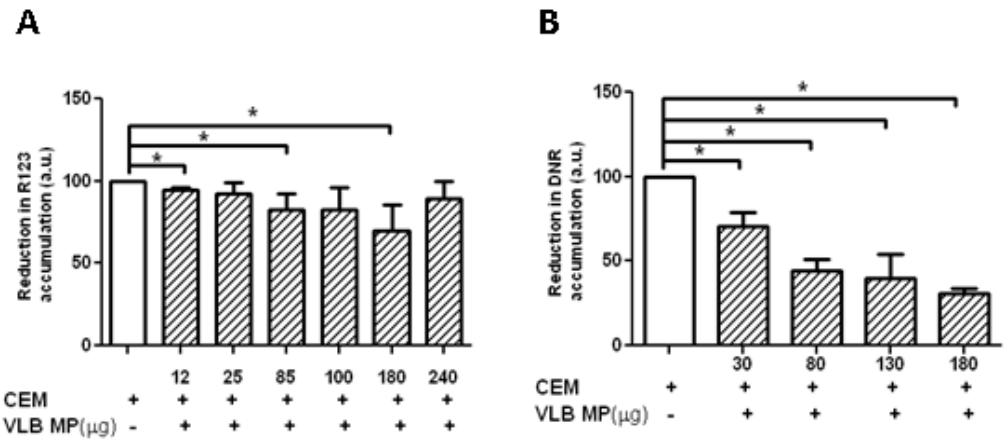

Figure 4: MP-mediated transfer of P-gp from drug resistance to drug sensitive cancer cells leads to the MDR phenotype. Reduction in whole cell accumulation of two fluorescent drug substrates of P-gp, namely, **(A)** Rh123and **(B)** DNR, in drug sensitive cells co-cultured with increasing amounts of MPs from drug resistant leukaemic cells. Values are expressed as percent reduction relative to the recipient drug sensitive cells. Data represent the mean ± SEM of at least 3 independent experiments *$p < 0.05$.

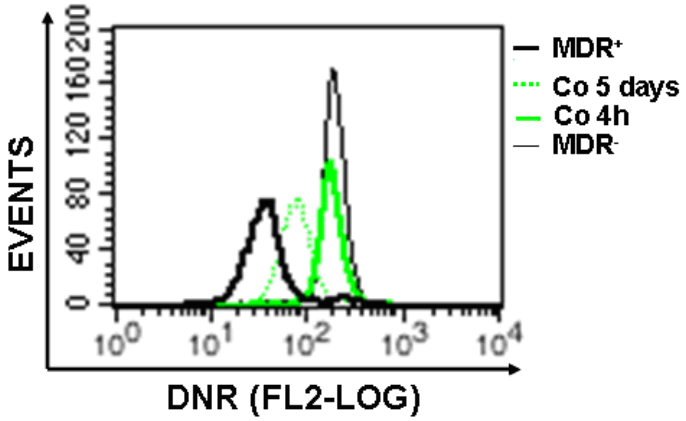

Figure 5: Acquired MDR phenotype stable *in vitro* for at least 5 days. MFI of DNR cell accumulation in a representative experiment. MDR^- cells (negative P-gp control), MDR^+ cells (positive P-gp control) and MDR^- cells co-cultured with MPs (MDR^+MP) from MDR^+ cells for 4 h, referred to as co-cultured cells (Co). MPs were removed by washing after 4hrs and cells left in culture for 5 days (Co 5days). The recipient cells post 4 h (Co 4h) and 5 days (Co 5days) were treated with 1μM DNR for 1hr at 37^0C and intracellular drug accumulation detected using flow cytometry. Figure shows DNR accumulation in — MDR^-, — Co 4h,···· Co 5 days and — MDR^+.