

Cell-Derived Microparticles: New Targets in the Therapeutic Management of Disease

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ABSTRACT - Intercellular communication is essential to maintain vital physiological activities and to regulate the organism's phenotype. There are a number of ways in which cells communicate with one another. This can occur via autocrine signaling, endocrine signaling or by the transfer of molecular mediators across gap junctions. More recently communication via microvesicular shedding has gained important recognition as a significant pathway by which cells can coordinate the spread and dominance of selective traits within a population. Through this communication apparatus, cells can now acquire and secure a survival advantage, particularly in the context of malignant disease. This review aims to highlight some of the functions and implications of microparticles in physiology of various disease states, and present a novel therapeutic strategy through the regulation of microparticle production.

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INTRODUCTION

Microvesicles are shed from most eukaryotic cells, and serve as vectors of long and short range signaling, facilitating the exchange of cargo between donor and recipient cells (1-4). Various microvesicles have been defined, including microparticles (MPs), exosomes and apoptotic bodies, with each vesicle type expressing distinct biochemical and structural characteristics (5, 6).

Microvesicle Classification

Microparticles

MPs are small enclosed plasma membrane fragments, measuring 0.1-1 μm in diameter, that typically express phosphatidylserine (PS) on their surface (2).

MPs are released from pre-apoptotic and activated cells by outward blebbing and vesiculation of the plasma membrane following a breakdown of the cytoskeleton, and play a central role in extracellular communication, inflammation, thrombosis, vascular function and oncogenic transformation (4, 7-13).

MPs are emerging as messengers for cells, whereby they can alter vascular function and induce various biological responses (14-16). In their role as messengers, MPs carry molecular components of the parent cell such as membrane

proteins, cytokines, integrins, transcription factors, nucleic acids and cytoplasmic contents, and can alter the activity of recipient cells through the transfer of their cargo (3, 4, 17, 18). This capacity has been implicated in long range cell signaling, coagulation, apoptosis, immune modulation, drug resistance and disease (19-24).

Apoptotic Bodies

Apoptosis leads to the formation of apoptotic bodies, which are membrane-enclosed cell fragments similar in size to platelets (1-5 μm in diameter) (25). These fragments consist of cytoplasm, organelles and nuclear fragments and are implicated in cancer progression and immunosuppression (26-29). Apoptotic bodies externalise PS to promote phagocytosis and removal (26, 30-32).

Exosomes

Exosomes are a smaller and more homogenous population of microvesicles, measuring 0.04-0.1 μm in diameter (approximately same size as viruses) that are released by the exocytosis of multivesicular

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bodies, and are formed by inward budding into the lumen at the endosomal limiting membrane (2, 33, 34). These multivesicular bodies fuse with the plasma membrane to release the small vesicles inside (the exosomes). Exosomes are released by most cell types, and contain cytosolic components, such as tetraspanin proteins, ribonucleic acids (RNAs), as well as cell surface proteins (2, 13, 35-38).

After being released from their respective cells, exosomes will either remain in the proximity of the cell of origin or relocate to biological fluids such as plasma, urine, milk, bronchoalveolar fluid and tumour effusions, and thereby facilitate long-range biological signaling (39-43).

Formation of Membrane-derived Microparticles

As platelet MPs have been the most widely studied vesicles thus far, the majority of information regarding MP shedding is in the context of activated platelets. The mechanisms involved however are believed to be similar for most cell types (44, 45).

Cell activation leading to MP formation is attributed to various stimuli such as serine proteases, thrombin, ADP, inflammatory cytokines (e.g. TNF), growth factors, shear and stress inducers (46-51). In contrast to this, apoptosis-induced MP release is regulated by the caspase-mediated Rho effector protein 'Rho-associated, coiled-coil containing protein kinase 1' (ROCK 1), and can also be stimulated by TNF and thrombin (50).

Disruption of Phospholipid Asymmetry

MPs are formed following disruption of the cell's phospholipid asymmetry (52-54). Under steady state conditions, phosphatidylcholine (PC) and sphingomyelin (SM) are located predominately on the outer membrane leaflet, and PS and phosphatidylethanolamine (PE) are located on the inner membrane leaflet (55, 56). This asymmetric distribution is under the control of the membrane-bound ATP-dependent enzymes; flippase and floppase, as well as the ATP-independent transporter scramblase (Figure 1) (57, 58). Flippase (the aminophospholipid translocase) rapidly directs phospholipids, specifically PS and PE, to the inner leaflet, whereas floppase promotes the much slower outward translocation of lipids (9). Scramblase is a bidirectional transporter and promotes random distribution of

phospholipids across the plasma membrane bilayer (56, 59).

Membrane reorganisation can occur under normal physiological conditions or in response to stimuli, such as following exposure to proinflammatory or prothrombotic substances, and involves the calcium-dependent inhibition of flippase and the promotion of scramblase and floppase activity (46, 56, 60). During this process, phospholipids, such as PS, are redistributed from the inner leaflet of the plasma membrane to the outer leaflet (9, 56, 61).

Membrane Vesiculation and the Role of Calpain

Following loss of phospholipid asymmetry, the anchorage between the membrane and cytoskeleton is disrupted. This allows for membrane vesiculation and subsequent MP release (45, 62).

In the case of apoptosis-induced MP release, the process is regulated via the activation of ROCK 1. During apoptosis, activated caspases (cysteine proteases associated with apoptosis) cleave ROCK 1, which promotes the generation of contractile force that leads to the formation of membrane blebs, disruption of the cellular membrane structure and subsequent vesiculation (9, 48, 63).

Conversely, the release of MPs from activated cells is associated with the activity of calpains. Calpains are intracellular cysteine proteinases, found in almost all eukaryotes and some prokaryotes, and play a central role in cellular functions such as cell cycle progression, gene expression, cytoskeleton cleavage, signal transduction, cell proliferation and MP formation (64-66). Calpain resides in the cytosol in its inactive form until an increase in cytosolic calcium translocates it to the cellular membrane where it is then activated (64).

After phospholipid scrambling, calpain cleaves cytoskeletal talin and α -actin filaments that adhere the plasma membrane to the cytoskeleton, thereby allowing the release of MPs (Figure 2) (53, 67, 68).

The calpain family consists of both tissue-specific and ubiquitously expressed isoforms. The best characterised isoforms are the ubiquitously expressed μ -calpain and m -calpain, which are named according to the concentrations of Ca^{2+} required to activate them *in vitro*; micromolar for μ -calpain and millimolar for m -calpain (69, 70).

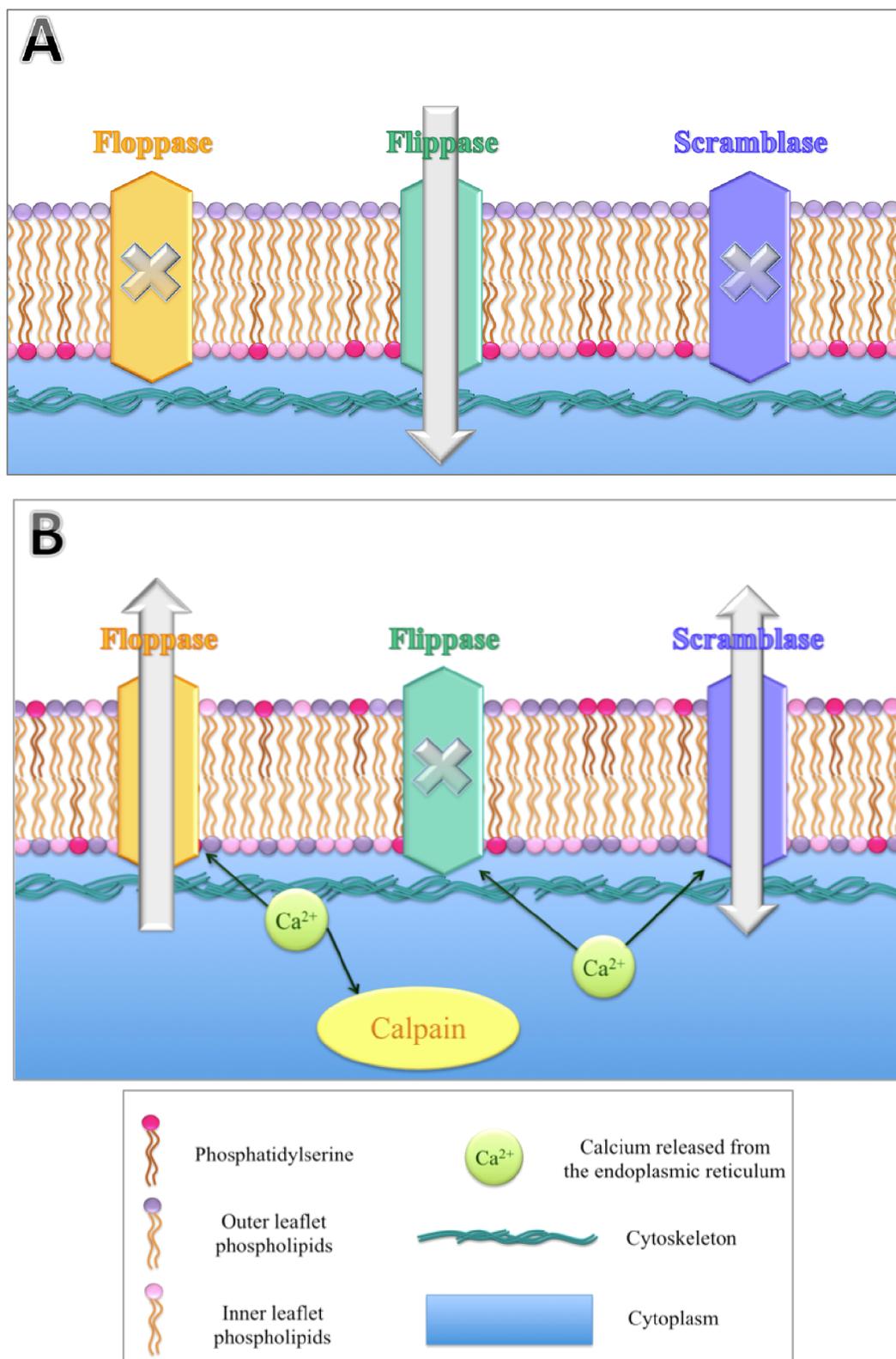


Figure 1. A) Schematic of a cellular membrane at rest. Phospholipid asymmetry is under the control of active flippase, whilst floppase and scramblase remain inactive. B) Cellular activation. Calcium is released from the endoplasmic reticulum, which can lead to the loss of phospholipid asymmetry and the activation of calpain.

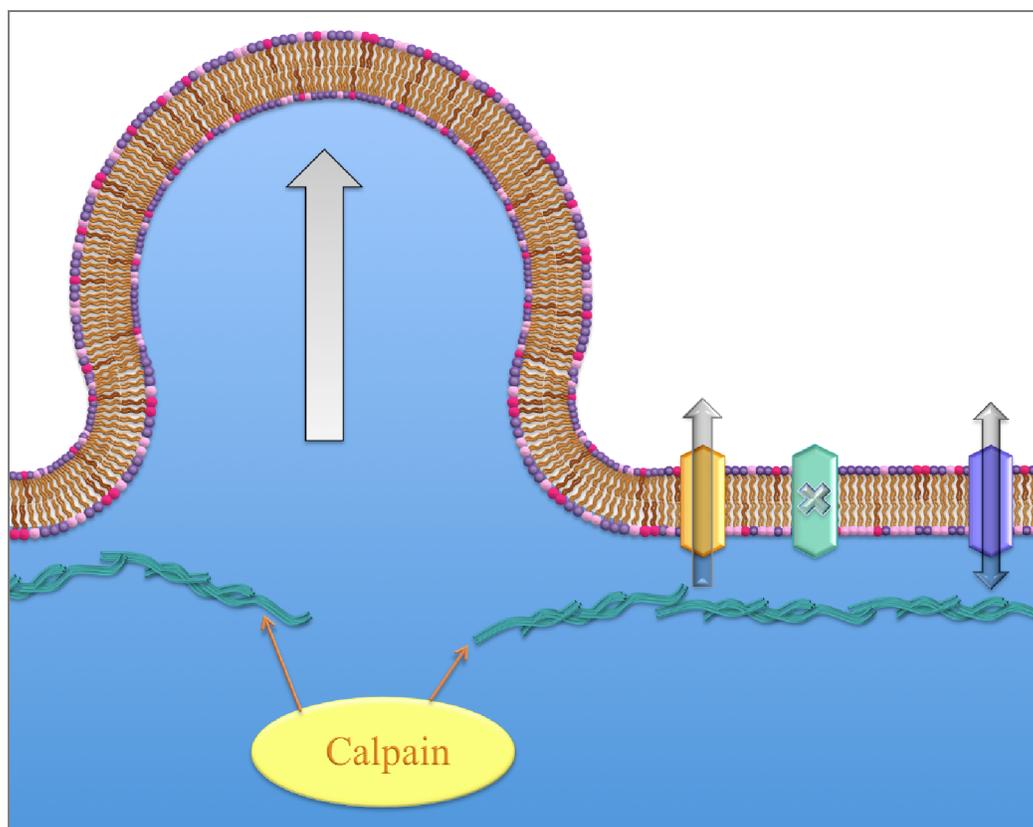


Figure 2. Cytoskeleton disruption. Activated Calpain cleaves the cytoskeleton, leading to the formation of a membrane bleb and subsequent MP release.

These proteins function as hetero dimers and are composed of large 80 kDa catalytic subunits and a small common 28 kDa regulatory subunit. μ -calpain is encoded by the gene *CAPN 1*, and *m*-calpain is encoded by *CAPN 2*, with the common regulatory subunit being encoded by *CAPN 4*. This small subunit is identical for both enzymes and the large subunits share 55-65% sequence homology (64, 69, 71).

Both μ -calpain and *m*-calpain can be subdivided into four domains. Domain II is the catalytic domain, conserved in all calpain isoforms, and is a triad site, which contains cysteine, histidine and asparagine residues. In human μ -calpain cysteine is located at residue 115, while in *m*-calpain it is located at residue 105 (69).

Calpain activity is tightly regulated by calpastatin, the endogenous inhibitor of μ -calpain and *m*-calpain and other dimeric calpains (65, 72). Calpastatin is a ubiquitously expressed inhibitor with four inhibitory domains, that displays specificity for calpain over other cysteine proteases, and can reversibly inhibit up to four

molecules of calpain at once by blocking the active sites of calpain (65, 69, 72).

The Role of Microparticles in Homeostasis

MPs play an important role in biological processes such as coagulation, intercellular communication, apoptosis and homeostasis (48, 73, 74).

Platelet and endothelial MPs circulating in blood express PS and tissue factor (TF) (the primary initiator of the extrinsic coagulation pathway) on their outer membrane. This exposed PS and TF provides a procoagulant surface for clotting enzymes in the coagulation cascade and therefore enables optimal thrombin generation (18, 75).

A crucial role for MPs in clot formation has been demonstrated *in vivo*, using a mouse model of haemophilia (76). In this setting, as well as in human blood, procoagulant MP were generated by the interaction of P-selectin and its natural ligand, PSGL-1.

MPs have been reported to express anticoagulant properties to balance their procoagulant activity. MPs indirectly down-regulate thrombin generation by promoting the generation of plasmin, and thus maintain vascular integrity (77-79).

MP-mediated cell signaling has also been implicated in the maintenance of homeostasis, with miRNAs that are predicted to regulate homeostasis and metabolic function being found in MPs circulating in the plasma of healthy donors (80).

Microparticles in Disease

Scott Syndrome

Reduced MP levels have been implicated in the physiological dysfunction seen in Scott syndrome, a very rare autosomal disorder, and the only human disease known to be caused by a lack of MP formation. This disorder is characterised by platelets, red blood cells and B-lymphoblasts lacking the ability to externalize PS and release MPs due to the abnormal expression of scramblase (2, 47, 57). The absence of PS exposure in this disorder results in severe bleeding due to impaired thrombin generation (47).

HIV-1

In a seminal study by Mack *et al.* it was shown that MPs disseminate specific chemokine receptors required for the propagation of HIV-1 to receptor-negative cells (20). In order to infect target cells HIV-1 requires the expression of CD4, an immune cell glycoprotein, and virus co-receptors which enable target cell binding and internalisation of viral particles (81). It was shown that various cell types release MPs containing the chemokine CCR5. These MPs are then able to transfer the receptor to CCR5⁺ cells and render them susceptible to HIV-1 infection. This transfer and acquisition demonstrated the important role that MPs play in the dissemination of deleterious traits (20).

Diabetes

Vascular disorder is closely linked to type 2 diabetes, with 50% of diabetic deaths being attributed to cardiovascular disease (82).

MP formation associated with type 2 diabetes has been implicated in the enhancement of thrombin generation and procoagulant activity,

with endothelial-derived MPs and monocyte-derived MPs reported to participate in the development of atherosclerosis in hyperlipidemic diabetic patients (83). With such a high death rate associated with symptoms secondary to the main disease, the modulation of MP release in the progression of diabetes could potentially help to circumvent the development of cardiovascular complications (83).

Cerebral Malaria

Malaria is a life threatening disease caused by *Plasmodium* parasites that is estimated to cause up to 1 million deaths per year (84).

Blood-borne *Plasmodium falciparum* is the most deadly species, and infection induces profound changes in the microvasculature, including TNF production, which leads to platelet adhesion, formation of endothelial-derived MPs, cerebral vascular inflammation and microcirculatory dysfunction (85-87).

Patients with severe malaria exhibit increased plasma levels of endothelial MPs. Remarkably, these high levels were seen in the presence of neurological complications – a syndrome called cerebral malaria (CM) – but not in patients presenting only severe malarial anaemia.

In CM, the major deleterious effects are not only attributed to the degree of infection itself, but to immune cell over-activation, resulting notably in cytokine storm, platelet activation and enhanced MP formation from various cellular origins (85, 87, 88).

In the experimental model for CM, ABCA1^{-/-} mice, which lack the floppase transporter required to externalise PS on MPs, were found to exhibit minimal vesiculation and complete resistance to CM, which suggests that MPs are directly implicated in the progression of the neurovascular pathology (14, 89, 90).

In CM, increased release of MPs correlated with the acute phase of the condition and is a potentially useful diagnostic tool and patient management aid, as MP levels associated with acute neurological phase returned to normal levels during recovery (88, 91).

Studies have concluded that the cellular origin, amount and composition of MPs can reflect the pathophysiological state and therefore could be used as diagnostic markers to monitor disease (91).

Cancer

Cancer-derived MPs were first reported in 1978 when they were detected in cultures from a patient with Hodgkin disease (92). Tumour cells can produce MP constitutively without any apparent need for stimulus but vesiculation can be increased by stress including exposure to chemotherapeutic drugs and heat (6).

Cancer patients not only exhibit tumour-derived MP but also high levels of platelet-derived MP (93). The hypercoagulation typically observed with malignancy can be attributed in part to these procoagulant MPs (94, 95).

MPs have been shown to contribute to tumour survival and it has been postulated that tumour cells can evade apoptosis by releasing MPs that contain caspase-3, thereby preventing its intracellular accumulation (96). Inhibition of this release has been shown to result in caspase-3 accumulation and subsequent apoptosis (96).

To facilitate invasion, metastatic cancer cells can degrade the extracellular matrix (ECM) through the transfer of surface proteases such as matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and cathepsins (97). Tumour cells have been shown to release MP rich in MMPs and uPA, which degrade the ECM, allowing for cell invasion (98).

Tumour-derived MPs also carry regulatory miRNAs, mRNAs and bioactive lipids, and can transmit them to other cells within the body (4, 6, 99, 100). Of particular concern is the MP assisted dissemination of cancer multidrug resistance, by which drug sensitive cells can acquire the resistance phenotype via long-range communication (3, 23).

Multidrug Resistance

Multidrug resistance (MDR) is the phenomenon by which cancers become cross-resistant to a wide variety of functionally and structurally unrelated chemotherapeutic drugs and constitutes the most significant obstacle in successful cancer treatment (100, 101).

MDR is predominately associated with the over-expression of the multidrug efflux transporters P-glycoprotein (P-gp/*ABCB1*) and Multidrug Resistance-Associated Protein 1 (MRP1/*ABCC1*), which are both expressed in the cell membrane of resistant cancer cells (102). These transporters belong to the superfamily of ATP-binding cassette (ABC) proteins which have also been implicated in infectious diseases such as AIDS and malaria (86, 89, 103, 104). P-gp and

MRP1 enable the maintenance of sublethal intracellular concentrations of chemotherapeutic drugs by facilitating the ATP-dependent efflux of anticancer agents, allowing the tumour to evade the toxic insult (100, 101, 104-107).

The mechanism that allows these transporters to bind and efflux a broad repertoire of drugs remains as yet undefined. Since the discovery in 1981 that verapamil and trifluoperazine could positively affect MDR by the modulation of P-gp, hundreds of drugs with different structures and activities, such as immunosuppressants, calcium channel blockers and steroids have been developed (108-110). Inhibitors of P-gp have been investigated in the prevention or circumvention of MDR clinically, with many showing positive effects *in vitro*, but proving to be unsuitable clinically due to dose limiting toxicity and lack of efficacy (111, 112).

Microparticle Mediated MDR

The over-expression of P-gp is known to be controlled by genetic and epigenetic mechanisms, and MDR acquisition through this process has been well characterised (113, 114). The spread of P-gp mediated MDR through non-genetic pathways however has been more recently reported, with direct membrane protein transfer occurring through direct cell-to-cell contact, shed MPs and tunneling nanotubes (23, 115).

'Non-genetic' acquisition of MDR was first demonstrated by Levchenko *et al.* through direct cell-to-cell contact (116). It was shown that P-gp could be transferred from P-gp positive cells to P-gp negative cells via direct cell-to-cell contact both in *in vitro* and *in vivo*, allowing previously drug-sensitive tumour cells to survive exposure to toxic levels of chemotherapeutic drugs. The acquired phenotype was transient *in vivo*, and required constant exposure to anti-cancer drugs or P-gp positive cells (116).

Our laboratory was the first to report that MDR can be spread in the absence of cell-to-cell contact with MPs being shown to facilitate the 'non-genetic' transfer of MDR from drug-resistant cells to drug-sensitive cells (23). We observed that MPs (i) were spontaneously shed from P-gp expressing leukemia cells (VLB₁₀₀), (ii) carry P-gp from the donor cells, (iii) bind to and transfer P-gp to recipient cells and (iv) confer the multidrug resistance phenotype to drug sensitive cells *in vitro* in as little as four hours following MP exposure (23).

Further investigation revealed that MDR breast adenocarcinoma cells (MCF-7/DX) also

spontaneously shed MP bearing functional P-gp and can confer the resistance phenotype to drug-sensitive recipient cells, demonstrating that cancerous cells of both haematological and non-haematological origin are able to transfer MDR via this pathway (100).

Validation of this MP mediated pathway was demonstrated *in vivo* using the MCF-7 murine tumour xenograph model. A single subcutaneous injection of P-gp positive MPs administered adjacent to the tumour mass, effectively resulted in the transfer of P-gp to the tumour core as early as 24 hours later. This acquired phenotype remained stable for at least two weeks without MP re-administration or exposure to a selective pressure (3).

In a recent study we also showed that MPs exhibit tissue selectivity, with respect to the transfer of P-gp to recipient cells. MPs derived from P-gp over-expressing breast adenocarcinoma cells (MCF-7/DX cells) selectively transferred P-

gp to malignant MCF-7 breast cells only. Conversely, MPs derived from P-gp over-expressing human leukaemia cells (VLB₁₀₀ cells) transferred P-gp and MRP1 to both malignant and non-malignant cells (3). This study demonstrates for the first time a tissue selective component in the transfer of MP cargo.

MP also mediate the transfer of regulatory nucleic acids from drug-resistant cells to drug-sensitive cells, and re-template the transcriptional landscape of recipient cells to reflect the donor cell (Figure 3) (4, 100). Further to this, we have shown that MPs transfer the transcripts that encode floppase and scramblase to recipient cells *in vitro*, as well as enzymes that are essential for miRNAs biogenesis (Drosha, Dicer and Argonaute) (4). The presence of these transcripts suggests that MPs could be capable of inducing vesiculation in recipient cells, as well as being key regulators of intercellular miRNA biogenesis.

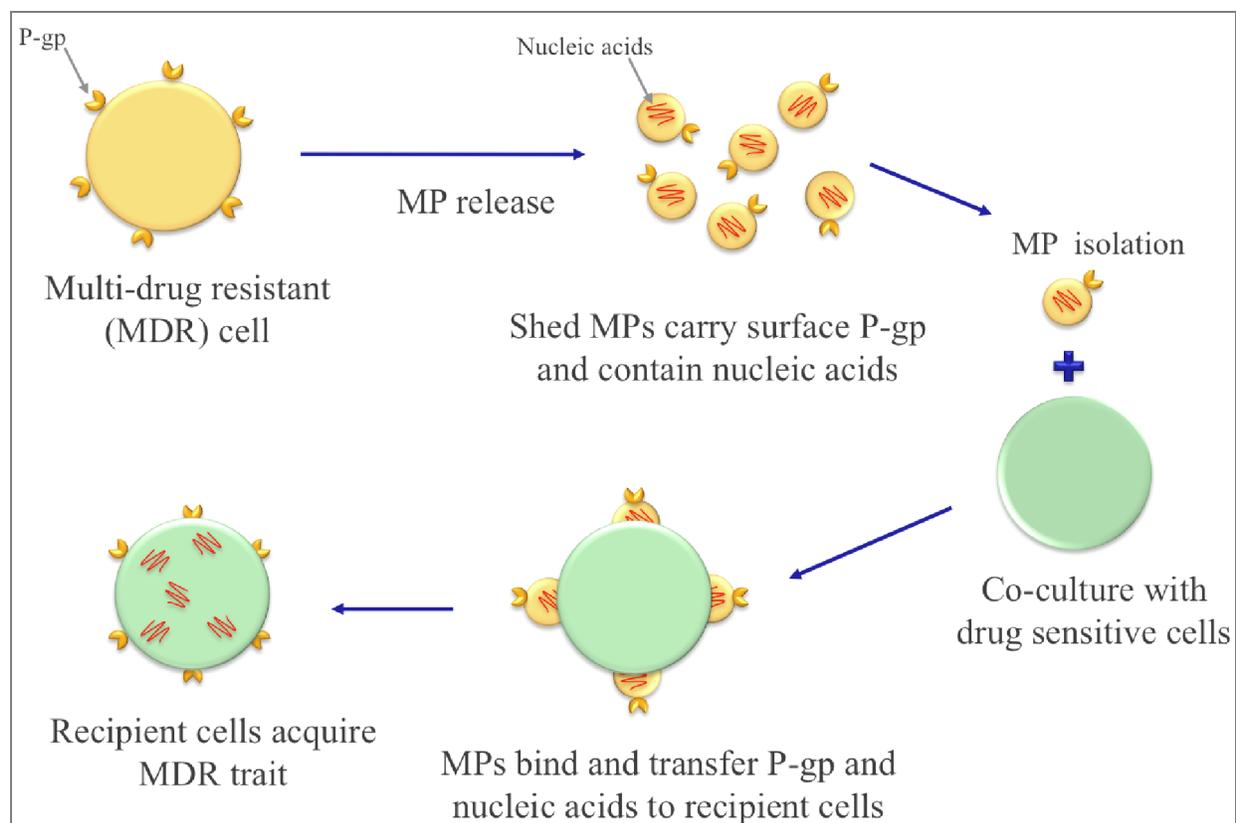


Figure 3. Mechanism for MP-conferred MDR *in vitro*. MPs, containing functional P-gp and nucleic acids, are released from multidrug resistant cells. MPs are then co-cultured with resistant cells. MPs confer MDR to drug-sensitive cells.

Microparticle Inhibitors

It has been well established that MPs actively contribute to the development and progression of numerous pathologies (2, 117). Modulation of their release therefore has the potential to provide a novel pharmacological strategy for disease state management.

There are currently a variety of inhibitors that have been identified as providing therapeutic potential in this regard. These include inhibitors of calpain activation, calcium channel blockers and various small molecule inhibitors.

Calpain Inhibitors

Due to the critical role calpain plays in MP release, targeting of calpain is a promising strategy to inhibit MP production. The development of potent and selective calpain inhibitors has received significant attention, as calpain has been implicated in several pathophysiological conditions including cerebral ischemia, Alzheimer's disease, Parkinson's disease, muscular dystrophy, thrombotic platelet aggregation, multiple sclerosis and diabetes, (68, 70, 118, 119). Unfortunately, most of this work has not examined the effects on MP production.

In the following section we provide a general outline of calpain inhibitors, and then focus on developments made in the context of inhibition of MP production.

Calpain inhibitors generally target the thiol-containing cysteine residue within the enzyme's catalytic domain. These inhibitors are broadly classified as reversible or irreversible depending on how they interact with cysteine, with both classes containing an electrophilic center, also referred to as a 'warhead'.

Reversible warheads include α -ketoesters, α -ketoamides and aldehydes, which all form a hemithioacetal or ketal with the cysteine thiol (120-122).

Irreversible warheads such as epoxides, α -haloketones, vinyl sulfone and diazomethyl ketone form irreversible covalent adducts (123-125). Irreversible inhibitors have received less attention in drug discovery due to the potential for unexpected or undesirable effects during treatment, as irreversible inhibition of calpain-activated cell-cycle progression, for example, could potentially have deleterious outcomes (118, 126).

In both classes, the warheads are commonly attached to a peptide backbone that is structurally

similar to endogenous substrates, thus assisting in binding to calpain's catalytic site and correctly positioning the warhead near the cysteine residue (127). A drawback to these peptidyl inhibitors is that rapid degradation by proteinases likely limits their *in vivo* efficacy. To circumvent this, several peptidomimetic inhibitors have been developed, however reactivity and metabolic instability of the warhead, as well as low calpain isoform selectivity remain issues for these inhibitor classes (128). Allosteric inhibitors of calpain have also been developed and show promise as selective inhibitors with improved pharmacokinetic properties (129, 130). These non-peptide inhibitors bind at the regulatory calcium-binding domain and prevent conformational changes required to activate calpain (69, 72).

As over-active calpain can induce aberrant MP release, potential inhibitors of its activity have been investigated in order to develop a novel treatment method for related disease states.

The calpain inhibitors MDL-28170 (a peptidyl aldehyde) and E-64-D (a peptidyl epoxide), (Figure 4) have been shown to significantly reduce MP release from platelets activated by agonists such as calcium ionophore A23187, thrombin and collagen (131). Likewise, calpeptin (200 $\mu\text{g/mL}$), a membrane-permeable inhibitor, has been shown to strongly inhibit the generation of MPs (~70%) from thrombin and collagen activated platelets *in vitro*, by inhibiting calpain activity as well as intracellular calcium elevation and attenuating thrombotic effects (67, 132). Due to their inhibitory activities these compounds have shown some potential as treatment options for MP-related conditions, however these compounds lack specificity for calpain (131, 133).

Thiosulfinates are another class of compounds that have been shown to inhibit calpain (134). These compounds occur naturally in different *Allium* species (such as onion and garlic), vegetables that have been shown to have beneficial health effects in conditions such as hypertension, inflammation, cardiac disease and cancer (135). Three members of the thiosulfinate family allicin, Pr₂TS and Me₂TS (Figure 4) were shown to inhibit calpain activation in platelets, leading to potent anti-aggregation activities with IC₅₀'s between 9 – 15 μM (119). Inhibition of aggregation was attributed to reduced calpain-mediated talin activation and reduction in platelet MP production: at 100 μM MP production was strongly inhibited.

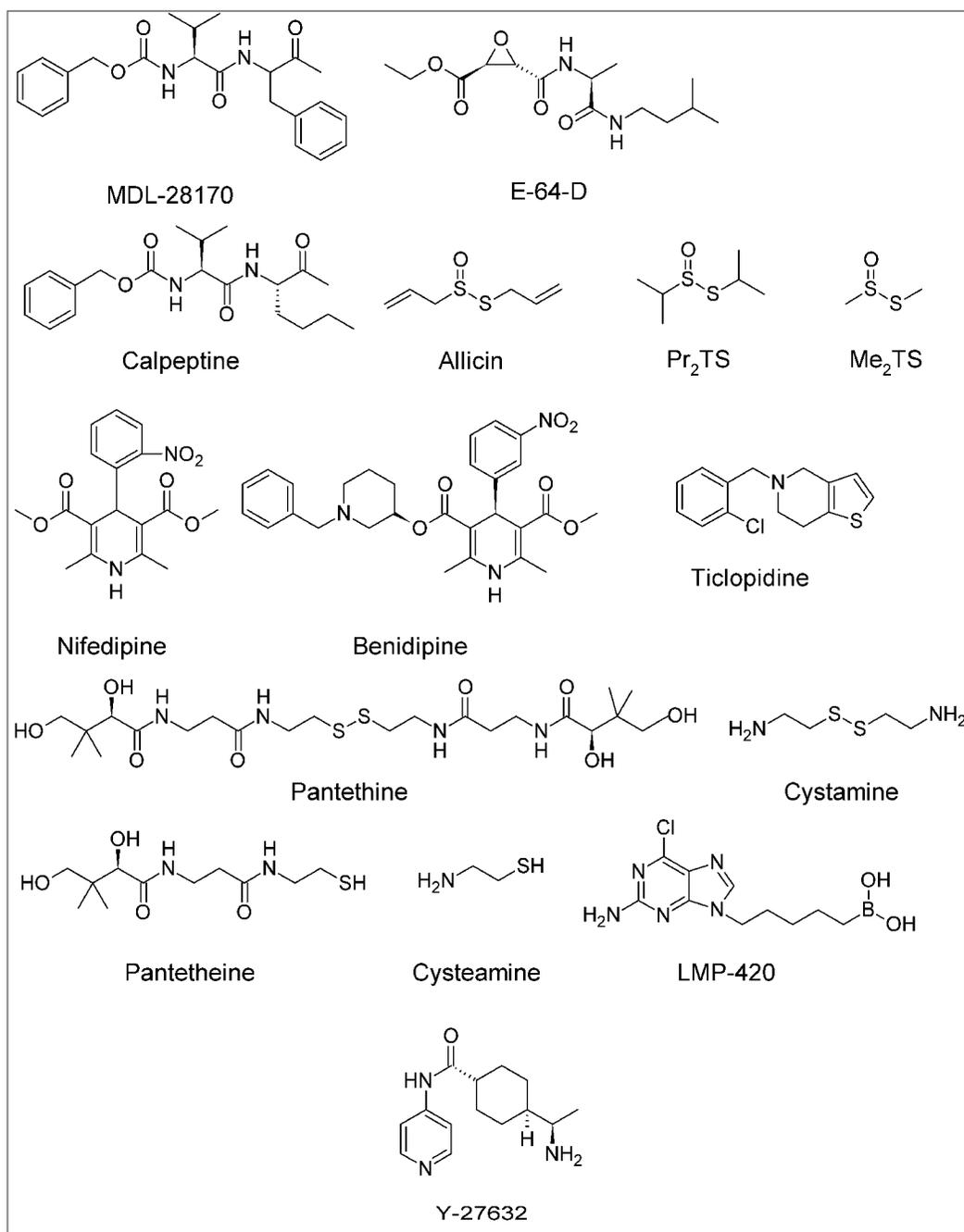


Figure 4. A selection of compounds reported to inhibit the formation of MPs

Calcium Channel Blockers

An increase in intracellular calcium plays a key role in MP generation, thus inhibition of cellular calcium influx is another strategy used to decrease MP production. Indeed, the calcium channel blockers nifedipine and benidipine, developed to treat hypertension, are effective in reducing MP

release by thrombin-activated platelets in patients suffering from transient ischemic attacks (TIA).

Elevated levels of circulating platelet MP are observed in patients with TIA and are associated with small vessel thrombosis (136, 137). Nifedipine (30-60 mg/day), a dihydropyridine calcium channel blocker (Figure 4), decreased cytosolic calcium concentrations and was shown to significantly reduce platelet MP levels (~50%)

in patients exhibiting TIA symptoms (138). As a short-term treatment, the administration of this drug to patients with TIA was shown to reduce the concentration of MPs in the circulation, however the drug did not appear to be a viable long-term treatment option as many of the patients suffered a recurrence of TIA whilst taking the drug (136, 138). Benidipine (4 mg/day), also a dihydropyridine calcium channel blocker (Figure 4), was administered over a six-month study to patients with diabetes mellitus to improve vascular dysfunction associated with monocyte and endothelial cell hypercoagulability. The administration of this drug resulted in decreased patient MP levels, and was shown to lower the blood pressure in hypertensive patients. (139). Whilst the outcomes for these drugs were positive, the patient MP levels were still much higher than in the healthy controls involved in the study (138, 139). This would suggest that whilst the effects are beneficial, further development of this class of drug is needed to effectively treat MP-associated symptoms.

Anti-platelet Drugs

Compared to healthy individuals, diabetic patients have increased levels of platelet-derived MPs (PDMPs) and monocyte-derived MPs (MDMPs), with both types of MPs promoting procoagulant activity (140). Ticlopidine (Figure 4), a thienopyridine antiplatelet drug used in the treatment of *arteriosclerosis obliterans*, has been identified for the treatment of hypercoagulability in patients with diabetes mellitus (83). The administration of 200mg/day to diabetic patients was shown to significantly reduce the number of systemic PDMPs (~30%) and MDMPs (~20%), demonstrating potential to prevent cardiovascular complications associated with elevated MP production (141). However, like calcium channel blockers outlined above, these drugs were not able to reduce the amount of MP to a level comparable to that in healthy individuals.

Pantethine/Cystamine

Pantethine (Figure 4), a disulfide intermediate in the production of Coenzyme A, inhibits platelet aggregation *in vitro* and has been shown to down-regulate TNF-activated MP release associated with CM, by modulating an early step of the inflammation-coagulation cascade (86). Mouse brain endothelial cells, incubated with 1 mM of pantethine for 24 hours showed a 51% reduction in MP formation, whilst *in vivo* mice treated with

pantethine had no signs of the associated neurological syndrome and exhibited MP levels similar to uninfected mice (86). Cystamine (Figure 4), another low molecular weight disulfide, also showed comparable inhibition of MP formation *in vitro*, but induced toxic shock with pathogenic consequences *in vivo* (86). In contrast, pantethine was well tolerated and showed potential as an effective therapeutic agent (86).

The monomeric analogues of these molecules, pantetheine and cysteamine (Figure 4), were also investigated but proved to be ineffective, indicating that the disulfide bond is necessary for inhibition. As yet, the mechanism of pantethine MP inhibition has not been characterised.

LMP-420

During CM, TNF-activated endothelial MP formation can be reduced by treatment with the low molecular weight purine compound (2-NH₂-6-Cl-9-[(5-dihydroxyboryl)-pentyl] purine), also known as LMP-420 (Figure 4). LMP-420 is a novel TNF- α inhibitor that produces strong anti-inflammatory effects and prevents cytokine-induced overproduction of MP release in response to activation by TNF- α as well as lymphotoxin- α (LT). MP release was shown to be significantly reduced in TNF- α or LT-activated human brain-derived endothelial cells (HBEC-5i) that were treated with 50nM of LMP-420 (142).

As MPs presenting in CM are inextricably linked with the inflammation-coagulation cascade, the inhibition of their formation could lead to a new therapeutic approach for increasing CM patient survival rates (142).

ROCK Inhibitors

Rho-associated protein kinases (ROCKs) regulate the actin cytoskeleton, and have been implicated in cancer cell migration, tumour progression and formation of tumour-derived microvesicles (143). In light of this, ROCK inhibitors, such as Y-27632 (Figure 4), have been developed as potential cancer therapies (144).

Y-27632, a low-molecular-weight inhibitor has been shown to reduce tumour-cell propagation *in vivo* and thus could potentially be used in cancer therapy (93, 145).

The treatment of HeLa cervical tumour cells, U87 brain tumour cells and MDA-MB-231 breast cancer cells with Y-27632 (5 μ M) was shown to significantly reduce the number of MPs produced through the ROCK-dependent signaling pathway

as well as eliminating the appearance of microvesicles on the cell surface (143).

To date no ROCK inhibitors are used in clinical treatments of cancer, however data suggests they have the potential to reduce primary tumour growth, as well as reducing metastasis (144).

CONCLUSIONS

Since their discovery, MPs have been shown to play a significant role in a wide variety of diseases and infectious conditions besides their important function in normal cellular processes. Due to these deleterious effects, and common occurrence in such conditions, the inhibition of their formation presents an attractive treatment strategy. Inhibitors developed thus far have shown some promise in this field.

Down-regulation of MP production in patients with diabetes has been shown to improve vascular dysfunction and lower blood pressure, whilst in mouse models, pantethine has been shown to reverse the effects of CM-related neurological syndrome.

As MP production is inextricably linked to cancer progression, inhibiting their formation has the potential to interfere with the ability of the cancerous cells to proliferate, as well as their ability to confer MDR to sensitive cells.

In light of the potential therapeutic effects of MP inhibitors, this area of research requires further development, as there are currently no specific or potent MP inhibitors available for the treatment of disease. Due to the widespread occurrence of aberrant MP release in many conditions, the development of MP inhibitors thus holds promise in providing a novel treatment paradigm across numerous pathophysiologicals.

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