Title

Mechanisms of drug resistance in cancer: The role of extracellular vesicles.

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List of abbreviations

ABC	– ATP binding cassette transporters
BE	– Bystander effect
BMSC	– Bone marrow Stromal cells
CAA	– Cancer associated adipocytes
CAF	– Cancer associated Fibroblasts
EVs	– Extracellular Vesicles
GIPC	– GAIP interacting protein C terminus
MDR1	– Multi drug resistance 1
MRP1	– Multidrug Resistance-Associated Protein 1
MVB	– Multivesicular body

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Abstract

Drug resistance remains a major barrier to the successful treatment of cancer. The mechanisms by which therapeutic resistance arises multifactorial. Recent evidence has shown that extracellular vesicles (EVs) play a role in mediating drug resistance. EVs are small vesicles carrying a variety of macromolecular cargo released by cells into the extracellular space and can be taken up into recipient cells, resulting in transfer of cellular material. EVs can mediate drug resistance by several mechanisms. They can serve as a pathway for sequestration of cytotoxic drugs, reducing the effective concentration at target sites. They can act as decoys carrying membrane proteins and capturing monoclonal antibodies intended to target receptors at the cell surface. EVs from resistant tumor cells can deliver mRNA, miRNA, long non-coding RNA and protein inducing resistance in sensitive cells. This provides a new model for how resistance that arises can then spread through a heterogeneous tumor. EVs also mediate cross-talk between cancer cells and stromal cells in the tumor microenvironment, leading to tumor progression and acquisition of therapeutic resistance. In this review, we will describe what is known about how EVs can induce drug resistance, and discuss the ways in which EVs could be used as therapeutic targets or diagnostic markers for managing cancer treatment. Whilst further characterisation of the vesiculome and the mechanisms of EV function is still required, EVs offer an exciting opportunity in the fight against cancer.

Introduction

In the past few decades there has been a large improvement in the effectiveness of cancer therapy, which is reflected in rising survival rates [1]. Despite these improvements, many patients will relapse with a tumor that is refractory to treatment, can metastasise to other tissues and severely reduces overall survival [2-6]. Our inability to prevent this stems from our incomplete understanding of the mechanisms by which tumor cells become resistant to the drug used to treat them. It is imperative, therefore, to fully understand the mechanisms of drug resistance so we can aim to prevent it arising, or reversing it once it has.

A major difficulty in understanding drug resistance is that the molecular mechanisms that underpin it are complex and multifactorial [7-11]. Indeed, resistance can arise via alterations in many different cellular processes, and the cause of resistance in one individual may not be the same as in another. For example, resistance can arise when proteins that export drugs from the cell are upregulated [12], or proteins that import drugs into the cell are reduced [13-16]. Such changes reduce the effective concentration of the chemotherapeutic at the active site, which is often the nucleus. Many chemotherapeutics work by inducing damage in DNA which leads to apoptosis in dividing cells; when cells gain the ability to repair the damage more effectively or they lose the ability to recognise the lesions and signal to the apoptotic machinery then this can cause resistance [17, 18]. Similarly, defects in the apoptotic machinery itself can render a tumor cell less capable of undergoing programmed cell death and thus more resistant to the effects of cytotoxic agents [19, 20]. Other changes can also bring about increases in resistance, such as altered autophagy activity [21], aberrant cell signalling activity, the unfolded protein response [22, 23] and epithelial to mesenchymal transition [24]. The acquisition of resistance by any of these mechanisms can be underpinned by mutations in the primary DNA sequence, deregulation of epigenetic marks and other changes in transcriptional processing such as aberrant RNA splicing or editing. Another factor which has emerged as important in the regulation of resistance is the role of intercellular communication, and in particular the role of extracellular vesicles (EVs) which will be the focus of this review.

EVs are small vesicular structures that are released by cells into the extracellular space. A wide variety of EVs have been described in the literature, including exosomes, microvesicles and apoptotic bodies (figure 1) [25]. Exosomes are produced when multivesicular bodies (MVBs), which contain intraluminal vesicles, fuse with the plasma membrane, releasing their vesicular contents into the extracellular region [26]. Microvesicles, on the other hand, are released when regions of the plasma membrane bud outwards from the cell before being pinched off as a fully formed vesicle [27, 28]. Apoptotic bodies are vesicles released by cells undergoing programmed cell death. Analysing EVs is challenging as there is a degree of overlap in the size and content of the different types of vesicle [25, 29]. Whilst we are beginning to understand the differences in vesicles based on their proteomic content the issue of vesicle heterogeneity is a major problem in the field [29-31]. For this reason isolated vesicles are grouped under the catch-all term EVs. Indeed, it is known that EVs can carry a range of cargo molecules, including miRNAs, long-non-coding RNAs (lncRNAs), mRNAs, and proteins [25, 32-34]. Extracellular vesicles were originally described as a means by which cells could remove unwanted cellular material [35]. However, it is now understood that EVs can be taken up by recipient cells via several uptake pathways and can deliver their cargo into the recipient cells [36]. This transfer of EVs from donor to recipient cells appears to be an important facet of the communication that occurs between cells and plays a role in many biological processes [25, 37-40]. It is not surprising, then, that they are deregulated in diseases and can play a role in many aspects of cancer progression, including in angiogenesis [41], proliferation [42], evasion of the immune response [43], regulation of metabolism, avoidance of apoptosis and metastasis [44].

EVs can also play a role in mediating drug resistance (figure 2). Cells that secrete more EVs show greater levels of resistance, possibly via release of drug which can be packaged into the vesicles [45]. EVs can also be released carrying cell-surface markers that are targeted by antibody therapies; these

EVs can act as decoys to bind antibody, thereby shielding the cells from the effects of the therapeutic [46]. Perhaps most intriguing is the numerous reports that describe how drug-resistant cells can transfer resistance from once cell to another, including from resistant to sensitive cells. This clearly has implications for the spread of resistance within a heterogeneous tumor. In this review we will describe the mechanisms by which EVs can transfer resistance to tumor cells.

EVs as a mechanism of resistance

Several studies have shown that cytotoxic drugs can be sequestered into EVs and released from the cell, thus preventing build-up of the drug in the nucleus. MCF7 cells (breast cancer) that are resistant to drugs over-express ABCG2 (an ABC transporter known to confer resistance to multiple drugs) which localises to EVs and mediates uptake of drugs into vesicles for release [47, 48]. This vesicular localisation of ABCG2 was dependent on PI3K/Akt signalling [47]. Correlation of a ‘vesicle shedding index’ (a score obtained by analysing expression of genes known to be contain in EVs) with patterns of resistance for a range of drugs demonstrated a link between EV output and doxorubicin resistance [49]. Further analysis shows that cells with higher indexes were better able to remove doxorubicin from the nuclear compartment and into the extracellular space (via EVs) [49]. Similarly, a drug-resistant subline of an ovarian cell line exported nearly three times as much cisplatin in their EVs compared to the sensitive parental cells [45]. Treatment of pancreatic cancer cells with gemcitabine enhanced release of EVs at a rate that was proportional to the measured sensitivity of the cells [50]. The increased production of EVs allowed more drug to be exported and thus the cells to be more resistant [50]. The orientation of some drug transporters may also be reversed in the membrane of EVs, which would allow these proteins to pump drugs into the vesicles rather than out, consistent with a role in mediating expulsion of the drugs in EVs and thus in mediating resistance [51]. Sequestration of cytotoxic compounds by EVs is therefore another potential mechanism by which tumors can gain resistance to therapy.

Fascinatingly, EVs may also act as decoys for antibody-based therapies. The use of monoclonal antibodies to target proteins and receptors at the surface of cancer cells is an expanding field. Whilst resistance to such therapies can arise through several routes, one emerging mechanism is the release of the targeted surface marker from the cell via EVs. For example, immunotherapy against CD20 can help to treat malignant lymphoma, but release of EVs bearing CD20 effectively shield the cells from antibody-mediated attack, in a process which required ABCA3, a drug transporter from the ABC (ATP-binding cassette) family of transporters[46]. Similarly, release of EVs carrying HER2 from cells which over-express this oncogene can interfere with the ability of Trastuzumab (the therapeutic antibody that binds HER2) to inhibit proliferation in breast cancer cells [52].

Proteomic analysis of EVs can give insight into mechanisms of drug resistance

The application of proteomic technology to analyse the protein content of EVs can give insight into the mechanisms by which EVs can cause resistance, and could also be used to identify biomarkers for the status of a tumour. A range of techniques can be used to analyse the vesiculome, including gel-based proteomics (in which proteins are separated on a gel and excised bands are analysed by mass spectrometry (MS), for example) and gel-free approaches (where total proteins are fragmented and analysed using techniques such as mass spectrometry) [53]. A comprehensive proteomic analysis of multiple cell types suggests that EV protein content closely reflects the cellular source of origin, suggesting that they offer a good indicator of cellular condition [54]. Exosomes released by apoptosis-resistant acute myeloid leukemia (AML) cells contain differences in their proteome, compared to those released by sensitive AML cells. Some of these proteins, which were related to processes including apoptosis and splicing, could be transferred between cells [55]. Proteomic analysis of EVs from prostate cancer cells that were sensitive or resistant to taxane revealed an upregulation of integrin β4 and vinculin in the EVs from resistant cells [56]. Similarly, another study showed that EVs from prostate cancer cells that were docetaxel resistance differed in their protein content compared to those released by sensitive cells. Interestingly MDR1, MDR3, Endophilin-A2 and PABP4 were up-regulated in EVs from resistant cells [57]. MDR1, MDR3 and PABP4 were also upregulated in the sera of a small sample of three patients with resistant prostate cancer [57], suggesting they could be used as a biomarker to predict treatment response. Proteomic analysis of EVs could also predict likelihood of side effects of treatment; one study showed that a 12-protein signature in serum EVs predicts the likelihood of chemotherapy-induced peripheral neuropathy in breast cancer patients [58]. Proteomics can therefore give an important insight into the mechanisms by which EVs could contribute to drug resistance.

Transfer of EV protein can cause transfer of drug resistance

In addition to their apparent role in mediating intrinsic resistance to drugs in cells, EVs have been observed to transfer resistance from one cell to another [59]. The transfer of vesicular protein between cells is one potential mechanism that could mediate these effects. The best characterised specific example is the transfer of P-glycoprotein (P-gp) between cells, with numerous groups around the world reporting their observations that P-gp transfer via vesicles can mediate resistance in recipient cells [60-70]. P-gp (*ABCB1*) is an ATP-binding cassette (ABC) transporter that can export drugs from cells with a broad range of specificity [71]. It is mainly expressed in epithelial cells lining organs such as the small intestine, colon and lungs, and it plays an important role in allowing these cells to act as a physiological barrier by pumping out xenobiotic and toxic substances [72]. Higher levels of P-gp (which can occur naturally, particularly in cancers arising from epithelial tissue at physiological barriers, or can occur after mutations or as a response to treatment [73, 74]) in tumors are associated with resistance to a range of compounds, and gives the gene its other name, Multidrug Resistance 1 (*MDR1*) [71]. Transfer of P-gp via EVs mediates resistance (for examples see table 1) and may also involve the action of other proteins that are transferred, such as Ezrin, Radixin, Moesin and CD44 [67]. Multidrug Resistance-Associated Protein 1 (*MRP1*) also known as *ABCC1* is a related drug efflux transporter that can also be transferred from resistant to sensitive leukaemia cells, conferring resistance in the recipient cell [75]. In other instances the P-gp protein is not directly transferred in EVs, but expression is induced in the recipient cell following delivery of a different protein. For example, MCF7 cells that are resistant to adriamycin transfer the protein TrpC5 (a Ca2+ permeable cation channel) to recipient cells via EVs, which then stimulates nuclear translocation of the NFATc3 protein and results in transcriptional activation of the *MDR1* (*ABCB1*) promoter [64]. It is likely that in future more examples will emerge of vesicular protein transfer that induces resistance in recipient cells.

Transfer of EV RNA can cause transfer of drug resistance

miRNA

EVs can carry a variety of coding and non-coding RNA cargo. miRNAs are short (19-25 nucleotides) non-coding RNAs that primarily function by binding to the RNA-induced silencing complex (RISC) and repressing gene expression via targeting of 3’UTRs of specific mRNAs [76, 77]. They can also function as ligands to RNA-binding proteins such as Toll-like receptors, leading to the activation of signalling pathways [78]. They have been implicated in stress response [79] and in cancer progression [76, 80, 81]. Importantly, their deregulation can promote drug resistance in tumors of different origins [82]. It is therefore not surprising that miRNAs transferred via EVs may play a role in mediating the transfer of resistance from one cell to another

Transfer of miRNAs can occur between docetaxel-resistant MCF7 cells and their sensitive parental line [83-85]. These miRNAs can cause downregulation of target genes in the recipient cells [83]. RNase treatment of the vesicles abrogates the transfer of resistance, suggesting RNA molecules are important in the mediation of the effect [83]. Transfer of miR-21, miR-27a and miR-451 from a chronic myeloid leukemia cell line to MCF7 cells led to increased resistance, possible via activation of Akt signalling [66]. EVs from a cisplatin-resistant ovarian cancer cell line (CP70) can transfer resistance to the sensitive parental line (A2780) via vesicular transfer of miR-214 and miR-21-3p, which induce resistance by repressing PTEN [86] and NAV3 [87], respectively. miRNA-155 in EVs from gemcitabine-resistant pancreatic cancer cells can induce resistance when taken up by sensitive recipient cells; miR-155 induces this resistance by targeting the pro-apoptotic gene TP53INP1 [88]. Transfer of miR-96 in lung cancer cells (from H1299 to A549 cells) may cause resistance to cisplatin that is mediated by targeting of the LIM-domain only protein 7 (LMO7) [89]. Another study with the same pair of cells showed that transfer of miR-222 may be involved in mediating resistance to adriamycin [90]. miR-221/222 can be transferred from tamoxifen resistant MCF7 to the sensitive parental cells leading to acquisition of resistance, possibly via targeting of P27 and ERα [91]. Taken together these studies show that transfer of extravesicular miRNA is one of the mechanism by which resistance can spread between cells.

<u>mRNA and long non-coding RNAs (lncRNAs)</u>

In addition to carrying miRNAs it is known that EVs can contain many other species of RNA, including coding mRNAs and lncRNAs. It is known that mRNAs can be transferred into recipient cells and actively translated into functional protein [32]. In addition to transferring P-gp protein to recipient cells, many resistant lines can produce EVs carrying *MDR1 (ABCB1)* or *MRP1 (ABCC1)* mRNA which can be transferred into sensitive cells to induce resistance [61, 66, 69, 75, 85]. A recent study highlighted the complexity of relationship between mRNAs and miRNAs delivered using leukaemia cell line CCRF-CEM and its multidrug resistant derivatives VLB_{100} and E_{1000} [92]. The authors showed that *ABCB1* (also known as *MDR1*), *ABCC1* (also known as *MRP1*) and miR-326 can all be delivered in EVs, but that expression of *ABCB1* dominates whilst miR-326 represses ABCC1. If *ABCB1* is knocked down then miR-326 represses *ABCC1* less efficiently, suggesting a complex interplay between RNA delivered in EVs.

LncRNAs are long transcripts that are not translated into proteins and are emerging as important regulators of function in cells [93, 94]. They have also been linked to cancer progression and acquisition of drug resistance in tumor cells [95]. Examples are emerging of lncRNAs mediating drug resistance via transfer in vesicles. The lncRNA linc-*ROR* can be upregulated by TGFβ or by chemotherapy, and when transferred via vesicles can induce resistance to sorafenib or doxorubicin in recipient HepG2 (hepatocellular carcinoma) cells [96]. Similarly the transfer of linc-*VLDLR* induces resistance in neighbouring hepatocellular carcinoma cells by increasing levels of the drug transporter ABCG2 [97]. Treatment of sensitive A2780 ovarian cells with EVs from resistant CP70 cells increased resistance in a miR-214-dependent manner [86]. Interestingly, treatment of the donor cells with curcumin (a key component of turmeric) caused up-regulation of the lncRNA *MEG3* which acted as a sponge to inhibit miR-214 and thus blunt the ability of CP70 EVs to induce resistance [86]. These results show that one of the mechanisms of EV-induced resistance is via the transfer of RNA species, including coding and non-coding RNAs.

<u>The role of lipids in vesicular resistance-transfer</u>

EVs are bound by a bilayer that contains a different balance of lipids compared to normal plasma membrane. They are enriched in various lipids, including ceramide, sphingomyelin, phosphatidylcholine, diacylglycerol and gangliosides [98, 99]. Interestingly, ceramide is required for EV biogenesis and is required for the loading of some of the cargo in vesicles [100, 101]. Ceramide has also been linked to drug resistance, possibly via P-gp activity and sequestration of drugs in EVs [40, 100, 102, 103]. A recent study of the lipidomics of EVs released by PC9R cells (a non-small cell lung cancer cell line resistant to Gefitinib) reveals a number of phospholipids that are over- or under-represented compared to the EVs released by the sensitive parental line PC9 [104]. Another study showed that artificial EV-like nanoparticles (composed of lipids similar to those found in EVs) could induce activation of NF-κB in MiaPaCa-2 cells (a pancreatic cell line) which then induces secretion of the chemokine SDF-1α [105]. This in turn interacts with the CXCR4 receptor on the surface of cancer cells and induces resistance via Akt signalling [105]. These results suggest that lipid biosynthesis and signalling may play a role in mediating resistance via transfer of EVs.

The role of the tumor microenvironment in EV-mediated transfer of drug resistance

Thus far we have discussed the way in which EVs transferred from resistant cancer cells can induce resistance when taken up by other, more drug-sensitive, cancer cells. However, tumor cells do not normally grow in isolation. In their native context cancer cells live alongside a variety of other cell types, which together are referred to as the tumor microenvironment [106]. Cross-talk between cancer cells and stromal cells has emerged as an important factor in the progression of a tumor [107]. A variety of stromal cells interact with cancer cells, including cancer associated fibroblasts (CAFs), tumor associated macrophages and endothelial cells. Indeed, the tumor microenvironment is now recognised as having at least as much complexity as any other organ. One of the methods by which cancer and stromal cells communicate is via the exchange of EVs. This EV-mediated communication, which occurs in a regulated fashion between epithelial cells and stromal cells, can become corrupted during tumorigenesis and can promote tumor progression and resistance to therapy [37, 108].

Numerous examples of EV transfer between stromal cells and cancer cells leading to resistance have now been documented. MSC exosomes could increase resistance of gastric cancer cells to 5'FU [109]. Treatment of colorectal cancer stem cells (either from patient xenografts or cell lines sorted for CD133) showed increased resistance (to chemotherapaeutic agents 5-fluorouracil and oxaliplatin) when treated with EVs from CAFs [110]. Stromal cells can transfer galectin-3 via vesicles to acute lymphoblastic leukaemia cells that induce endogenous production of further galectin-3 and NF-kB activation which leads to drug resistance [111]. Bone marrow stromal cells (BMSCs) release EVs that can induce resistance to bortezomib in multiple myeloma cells [112]. This may involve activation of survival signalling pathways including JNK, p38, p53 and Akt [112]. RNA in exosomes from stromal cells can induce resistance in breast cancer cell by triggering signalling via the pattern recognition receptor RIG-I and via NOTCH3, leading to the activation of a STAT1-dependent antiviral response [113].

A common theme in the acquisition of drug resistance via EV transfer within the tumor microenvironment is the transfer of functional miRNAs [114]. For example, cancer associated adipocytes (CAAs) and fibroblasts (CAFs) transfer vesicular miR-21 to ovarian cancer cells leading to increased paclitaxel resistance via targeting of the apoptosome component APAF1 [115]. Treatment of CAFs with gemcitabine leads to increased release of EVs with the capacity to induce resistance in pancreatic cancer cells by transferring mRNA encoding the resistance factor Snail and also miR-146a [116]. Mesenchymal stem cells primed by interaction with breast cancer cells release EVs with miR-222/223 that induce quiescence in a proportion of the cancer cells and induce drug resistance [117]. Bone marrow mesenchymal cells taken from human donors were able to repress proliferation and increase resistant bone marrow-metastatic cell line derived from MDA-MB-231 breast cancer cells [118]. The mechanism involved transfer of miR-23b into the recipient cells which repressed the cell cycle regulator MARCKS [118]. Interestingly, EV-mediated communication can occur in both directions. For example, neuroblastoma cells release EVs with miR-21 which interact with the Toll-like receptor in monocytes, leading to reciprocal release of EVs from monocytes carrying miR-155, which in turn enter neuroblastoma cells and repress TERF1 to induce cisplatin resistance [119]. These results demonstrate that EV-mediated cross-talk within the tumor microenvironment is an important mechanism by which resistance can be transferred to cells.

Potential role of EV-mediated bystander effects

Another interesting factor in drug resistance is the role of EVs during stress response which could lead to bystander effects. The bystander effect (BE) is a phenomenon in which cells undergoing stress can communicate with other cells to coordinate an intercellular response [120]. The BE was originally described using radiation; when cells are exposed to ionizing radiation they release a secreted signal which can induce DNA damage when placed onto naïve cells [121]. This effect has also been observed following treatment of cells with other stress types, including cytotoxic chemicals [122, 123] and heat stress [124]. We have recently shown that the BE is mediated by release and uptake of EVs following irradiation[125], cytotoxic stress [127] or heat [128]. The observation that BE is evolutionarily conserved implies a potential role in organismal fitness. One potential benefit to the BE is that the bystander cells, although apparently stressed, are in fact more robust in the face of future insults. In other words, EVs released from stressed cells can induce an adaptive response in naïve recipient cells[127-130]. Applied to the cancer therapy setting one could imagine a situation where in a cohort of tumor cells treatment with radio- or chemotherapy could induce release of EVs into the extracellular space which could induce an adaptive response within the population. Evidence of such a model does exist in the literature. For example, treatment of A549 cells with EVs released by cisplatin-treated A549s increased resistance to cisplatin [131]. Treatment of pancreatic cells (MiaPaCa and Colo-357) with gemcitabine led to release of EVs with capacity to induce resistance to gemcitabine [132]. This was partly mediated by transfer of miR-155 which represses DCK (a gemcitabine metabolising enzyme) and transfer of SOD2 and CAT which can both detoxify reactive oxygen species [132]. Treatment of hepatocellular carcinoma induces upregulation of lncRNAs in EVs that can cause resistance when placed onto naïve cells [96, 97]. EV-mediated communication during chemotherapy could therefore also be a mechanism by which resistance arises within the tumor mass.

Perspective and future directions

The body of work reviewed here provides a compelling case for the role of EVs in mediating drug resistance. However, many questions remain unanswered. The full range of mechanisms by which EVs induce resistance has certainly not been fully elucidated, so further work is necessary. For example, in some studies a role for activation of signalling pathways such as HGF/c-Met/Akt can be established [133], but not the precise mechanism by which they become activated. In some cases there is also contradictory evidence; knockdown of *MDR1 (ABCB1)* in donor cells does not always have an effect on the transfer of resistance, suggesting that either the role of *MDR1*/P-gp *(ABCB1)* transfer is complex, or that this particular transfer represents one of multiple mechanisms by which induction of resistance can be achieved by EVs [109]. Other studies suggest that transfer of EVs does not always lead to increased resistance. For example, in one study using androgen independent prostate cancer cell line DU-145, transfer of EVs from resistant cancer cells to their parental line did not induce resistance [57], whereas other studies showed that DU-145 EVs induce resistance [42, 61]. These conflicting results may be due to subtle differences in methodology such as drug concentration used, incubation time or thresholds set, the type of drug resistance being tested, or could suggest that EVs have context-dependent effects. For this reason it is important that all results of EV-transfer experiments are published and that 'negative' results are not excluded from the literature.

Many of the examples described in this review revolve around the role of miRNAs. However, important questions remain surrounding the association of EVs with miRNAs. Some studies show that a large proportion of miRNAs released extracellularly into the culture media or circulation may in fact be non-vesicular and instead associated with other proteins such as Ago2 [134, 135]. Indeed, recent stochiometric measurements of vesicular miRNAs suggest that most EVs do not actually carry any miRNAs [136]. This raises the question of whether the numerous reports of EV-mediated transfer in fact represent transfer of non-vesicular material (which may co-purify with EVs), whether the EVs do not contain miRNAs luminally but contain the miRNA associated at the vesicular surface, or whether further quantification is required to determine the stoichiometry of vesicular miRNA. More work is certainly needed to explore these questions further.

Potential for therapeutic and prognostic applications

Better understanding of the way EVs mediate drug resistance could lead to novel combination treatments that are more effective. EVs appear to mediate bystander effects that occur following exposure of cells to radiation or cytotoxic drugs; given that these effects include an adaptive response it should be possible to sensitise cells to treatment if EV transfer is inhibited. Indeed, treatment of cells with heparin (an EV uptake-inhibitor) can sensitise cells to cisplatin [137], and our data suggest that this may be due to the inhibition of EV uptake [127].

The vesicular sequestration and expulsion of cytotoxic drugs as a means to achieve resistance could be targeted by combination therapies. Targeting the drug transporter ABCA3, another member of the ABC family of drug transporters, (using either shRNAs or the COX inhibitor indomethacin) reduced exosome biogenesis and increased nuclear retention (and thus effectiveness) of doxorubicin and pixantrone [138]. Treatment of cells with guggulsterone (a farnesoid X receptor antagonist) and bexarotene (a retinoid X receptor agonist) induced higher levels of ceramide which in turn stimulated the release of EVs. These EVs appear to carry ABC transporters (breast cancer resistance protein BCRP/ABCG2) which is then depleted from the cell and increases the sensitivity of these cells to subsequent doxorubicin treatment [103]. Knockdown of GIPC (GAIP interacting protein C terminus) induces autophagy and the release of EVs with elevated levels of ABCG2 [139]. Following release of these EVs the cells are more sensitive to gemcitabine, though it is possible this is due to the pleiotropic effects of GIPC knockdown [139]. Treatment of tumor cells with a proton-pump inhibitor alters pH and induces the release of EVs leading to increased sensitivity to cisplatin [140]. Taken together these findings suggest that inhibiting or affecting the compartmentalisation of therapeutics into EVs can lead to redistribution and/or accumulation of drugs in cells, with potential increased efficacy of treatment.

Greater understanding of the roles of miRNAs could also be used to target EV-mediated resistance. For example, when tumor cells were treated with a curcumin the level of lncRNA *MEG3* in these cells rose and appeared to sequester miR-214, thus reducing the ability of vesicular miR-214 to induce resistance in sensitive cells [86]. Treatment of miRNA inhibitors achieved a similar result, suggesting that pharmaceutically targeting specific miRNAs could block the spread of resistance in cancer [86]. An alternative approach is to isolate vesicles from cells artificially over-expressing a miRNA or anti-miR that, when transferred via EVs, can induce sensitivity in recipient cells. For example, EVs from adipose-derived mesenchymal stem cells over-expressing miR-122 (driven by an expression plasmid) can sensitise hepatocellular carcinoma cells to drug treatment [141]. Similarly, EVs from mesenchymal stem cells transfected with an anti-miR-9 can induce sensitivity to temozolomide when taken up by glioblastoma cells [142].

It is also noteworthy that EVs and their content can serve as biomarkers for cancer [143-147]. Given that there are changes in EV content that could mediate their ability to induce drug resistance when transferred between cells it is logical that these changes could also serve as a biomarker for the presence of tumors that are either resistant to specific drugs or harbour the capacity to transfer resistance [148]. Indeed, many studies have been published aiming to characterise differences in EVs from sensitive/resistant cultured cancer cells [33, 90, 104, 149] or from the blood of patients with different responses to therapy [150]. Further development and use of these biomarkers would then allow clinicians to either alter the therapy choice or, eventually, target both cancer cells and the EV-mediated communication. Better characterisation of the extracellular vesiculome and greater understanding of the roles of EVs will help to unlock their therapeutic and prognostic potential.

Final remarks

It has become very clear in the last few years that EVs are an important part of the dialogue that occurs between different cells [25]. Tumor cells also release EVs, allowing them to transfer macromolecules to other cancer cells or stromal cells in the tumor microenvironment. This transfer can have a range of phenotypic effects, but ultimately many of these support the growth of the tumor. Importantly, it can lead to the transfer of resistance from one tumor cell to another. One can envisage how this would allow resistance across a population of cells in a tumor mass to rise, as resistance could be acquired in a pocket of the heterogeneous mass and then spread to other cells via the release of EVs. This, then raises the possibility that drug resistance may be modulated by locally inhibiting EV release or uptake. This offers an exciting new insight into the biology of cancer, and offers potential therapeutic targets for tackling the acquisition of therapeutic resistance. Further work must be performed, particularly *in vivo*, to better elucidate the mechanisms by which transfer of EVs can induce resistance.

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Figure Legends

Figure 1: Types of Extracellular vesicles Microvesicles are formed by outward budding of the plasma membrane which is then pinched off into the extracellular space. Exosomes are formed by invagination into multivesicular bodies as intraluminal vesicles; these are released by fusion of the multivesicular bodies with the plasma membrane. Apoptotic bodies are formed during blebbing of the plasma membrane when cells undergo apoptosis.

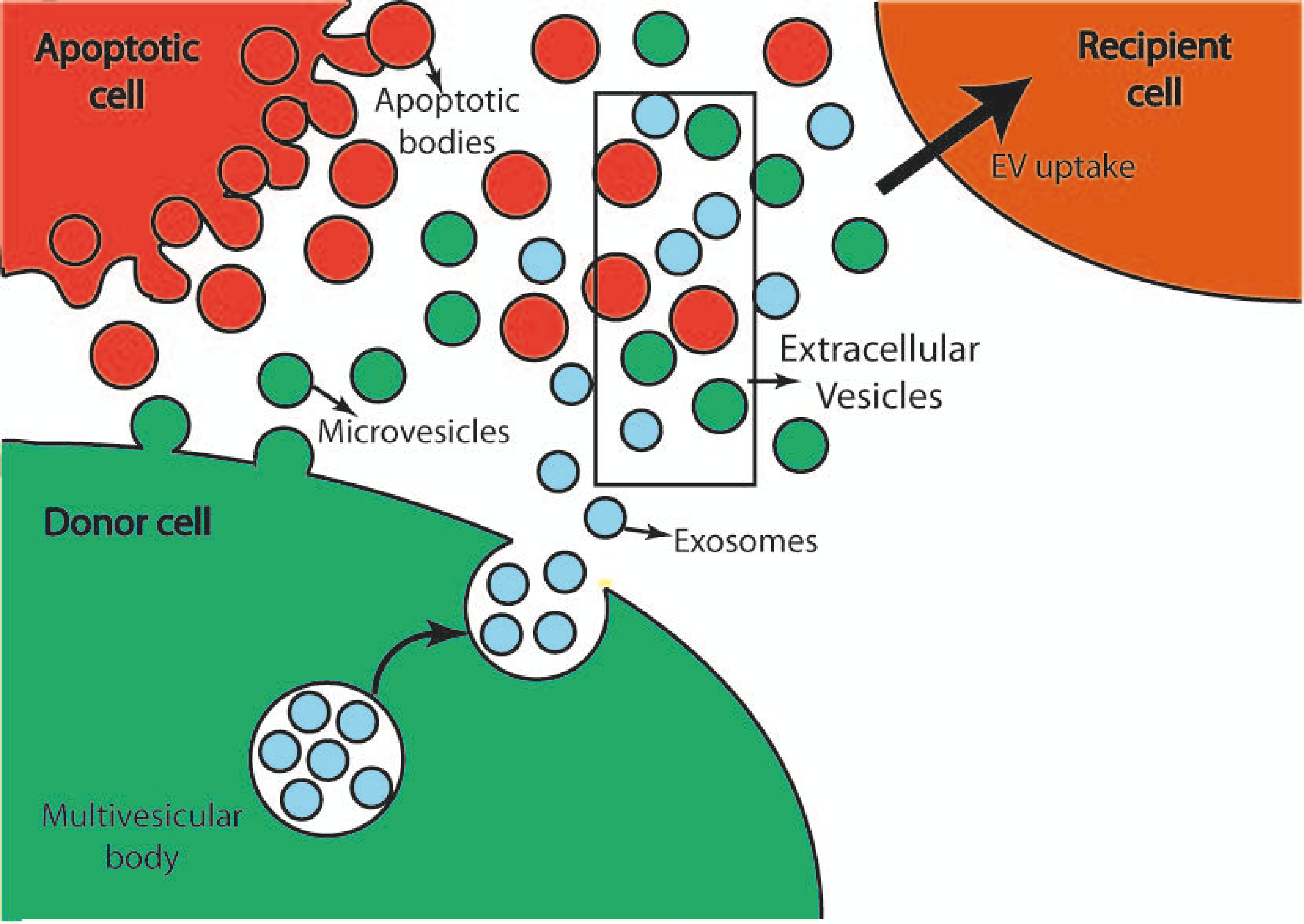
Figure 2: Mechanisms by which extracellular vesicles are shown to modulate response to cancer chemotherapy EVs have been shown in various studies to modulate chemoresistance in cancer cells. It has been demonstrated that drugs may be sequestered in extracellular vesicles thereby decreasing the effectiveness of the drug. Another EV-based resistance mechanism involves resistance to immunotherapy – EVs presenting CD20 on their surface have been shown to act as a decoy, thereby shielding the cell from immunotherapy using antibodies to CD20. Various studies have shown transfer of a number of proteins, mRNA, lncRNA, miRNAs and lipids from resistant cells to recipient cells, thereby bequeathing a degree of drug resistance. The most common of these is the transfer of drug efflux proteins such as P-gp (ABCB1) and MRP1 (ABCC1) or mRNA encoding these. Several microRNAs shown to be transferred through EVs are also implicated in increasing drug resistance in the recipient cells. Another important factor are the EVs released by stromal cells which have been shown to modulate resistance to cancer chemotherapy by transferring proteins, miRNA and mRNA.

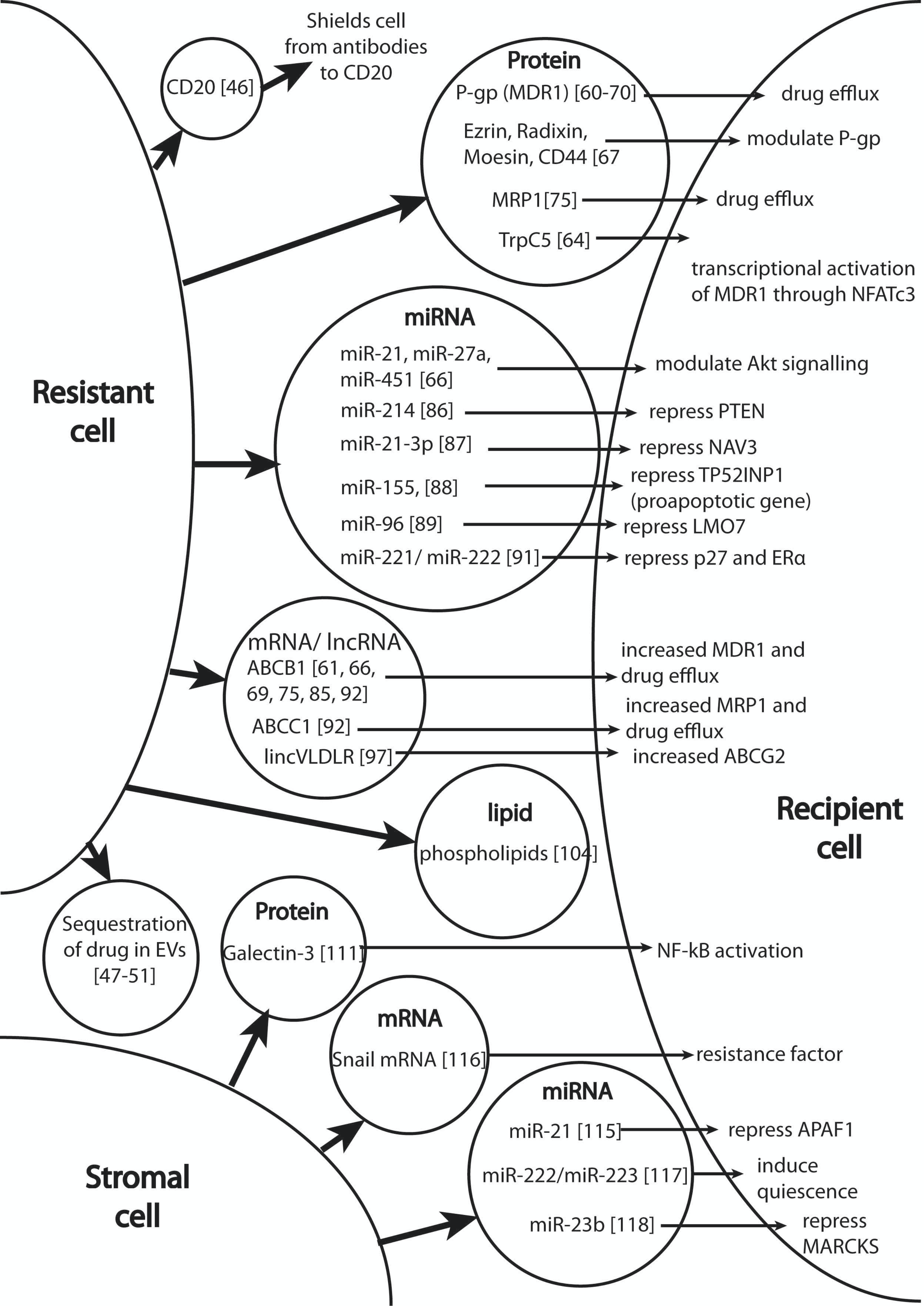
Tables

Table 1: Studies showing proteins or mRNA transferred through extracellular vesicles

tissue	donor	recipient	mRNA/ protein	drug	First author and reference
leukaemia	VLB100	CCRF-CEM	P-gp / ABCB1 protein	Multidrug resistant	Bebawy M [62]
leukaemia	VLB100	CCRF-CEM	P-gp/ ABCB1 protein	Multidrug resistant	Jaiswal R [63]
breast	MCF-7DX	MCF-7	P-gp/ ABCB1 protein	Multidrug resistant	Jaiswal R [63]
breast	MCF-7ADM	HMEC	P-gp/ ABCB1 protein	adriamycin	Dong Y [64]
breast	MCF-7ADR	MCF-7	P-gp/ ABCB1 protein	adriamycin	Wang X [68]
ovarian	A2780/PTX	A2780/WT	P-gp/ ABCB1 protein	Paclitaxel and adriamycin	Zhang FF [60]
prostate	DU145RD and 22Rv1RD	DU145 and 22Rv1	P-gp/ ABCB1 protein	docetaxel	Corcoran C [61]
breast	MCF-7/DOXO	MCF-7	P-gp/ ABCB1 protein	doxorubicin	Pasquier J [65]
haematopoetic	K562 MDR variant - Lucena cell line	A549 and MCF-7	P-gp/ ABCB1 protein and mRNA	Multidrug resistant Cisplatin, paclitaxel	de Souza PS [66]
breast	MCF7 DX	MCF-7	P-gp/ ABCB1 protein	Multidrug resistant	Pokharel D [67]
breast	MCF-7/DOC	MCF-7	P-gp/ ABCB1 protein	docetaxel	Lv MM [70]
Breast, leukaemia	VLB$_{100}$, MCF-7/Dx	CCRF-CEM, MCF-7	Ezrin	Multidrug resistant	Pokharel D [67]
Breast, leukaemia	VLB$_{100}$, MCF-7/Dx	CCRF-CEM, MCF-7	Radixin	Multidrug resistant	Pokharel D [67]
Breast, leukaemia	VLB$_{100}$, MCF-7/Dx	CCRF-CEM, MCF-7	Moesin	Multidrug resistant	Pokharel D [67]
Breast, leukaemia	VLB$_{100}$, MCF-7/Dx	CCRF-CEM, MCF-7	CD44	Multidrug resistant	Pokharel D [67]
Leukaemia	E$_{1000}$, VLB$_{100}$	CCRF-CEM	MRP1/ ABCC1 protein	Multidrug resistant	Lu JF [75]
Breast, Leukaemia	VLB$_{100}$, MCF-7/Dx	CCRF-CEM, MCF-7	TrpC5	Multidrug resistant	Pokharel D [67]
Leukaemia, stroma	OP9 stromal cells	TXL2, US7	Galectin-3	Vincristine and BMS345541	Fei F [111]
leukaemia	VLB$_{100}$ and MCF-7 DX	CEM and MCF-7	ABCB1 mRNA	Multidrug resistant	Jaiswal R [85]
leukaemia	VLB$_{100}$	CEM	ABCB1 mRNA	Multidrug resistant	Lu JF [75]
leukaemia	VLB$_{100}$	CEM	ABCB1 mRNA	Multidrug resistant	Lu JF [92]
Leukaemia	E$_{1000}$	CCRF-CEM	ABCC1 mRNA	Multidrug resistant	Lu JF [92]

Pancreatic cancer	CAF1	L3.6	Snail mRNA	Gemcitabine	Richards KE [116]


Apoptotic cell
Apoptotic bodies
Recipient cell
EV uptake
Extracellular Vesicles
Microvesicles
Donor cell
Exosomes
Multivesicular body


Resistant cell
CD20 [46]
Shields cell from antibodies to CD20
Protein
P-gp (MDR1) [60-70]
drug efflux
Ezrin, Radixin, Moesin, CD44 [67
modulate P-gp
MRP1[75]
drug efflux
TrpC5 [64]
transcriptional activation of MDR1 through NFATc3
miRNA
miR-21, miR-27a, miR-451 [66]
modulate Akt signalling
miR-214 [86]
repress PTEN
miR-21-3p [87]
repress NAV3
miR-155, [88]
repress TP52INP1 (proapoptotic gene)
miR-96 [89]
repress LMO7
miR-221/ miR-222 [91]
repress p27 and ERα
mRNA/ lncRNA
ABCB1 [61, 66, 69, 75, 85, 92]
increased MDR1 and drug efflux
ABCC1 [92]
increased MRP1 and drug efflux
lincVLDLR [97]
increased ABCG2
lipid
phospholipids [104]
Recipient cell
Sequestration of drug in EVs [47-51]
Protein
Galectin-3 [111]
NF-kB activation
mRNA
Snail mRNA [116]
resistance factor
miRNA
miR-21 [115]
repress APAF1
miR-222/miR-223 [117]
induce quiescence
miR-23b [118]
repress MARCKS
Stromal cell