

1 **Title**

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

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15 **List of abbreviations**

16 ABC – ATP binding cassette transporters

17 BE – Bystander effect

18 BMSC – Bone marrow Stromal cells

19 CAA – Cancer associated adipocytes

20 CAF – Cancer associated Fibroblasts

21 EVs – Extracellular Vesicles

22 GIPC – GAIP interacting protein C terminus

23 MDR1 – Multi drug resistance 1

24 MRP1 – Multidrug Resistance-Associated Protein 1

25 MVB – Multivesicular body

26 **Keywords:** Extracellular vesicles, drug resistance, Cancer, microRNAs

27 Total number of words: 8577

1 **Abstract**

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

18

1 **Introduction**

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

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

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

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

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

6 **EVs as a mechanism of resistance**

7 Several studies have shown that cytotoxic drugs can be sequestered into EVs and released from the
8 cell, thus preventing build-up of the drug in the nucleus. MCF7 cells (breast cancer) that are resistant
9 to drugs over-express ABCG2 (an ABC transporter known to confer resistance to multiple drugs)
10 which localises to EVs and mediates uptake of drugs into vesicles for release [47, 48]. This vesicular
11 localisation of ABCG2 was dependent on PI3K/Akt signalling [47]. Correlation of a 'vesicle shedding
12 index' (a score obtained by analysing expression of genes known to be contained in EVs) with patterns
13 of resistance for a range of drugs demonstrated a link between EV output and doxorubicin resistance
14 [49]. Further analysis shows that cells with higher indexes were better able to remove doxorubicin
15 from the nuclear compartment and into the extracellular space (via EVs) [49]. Similarly, a drug-
16 resistant subline of an ovarian cell line exported nearly three times as much cisplatin in their EVs
17 compared to the sensitive parental cells [45]. Treatment of pancreatic cancer cells with gemcitabine
18 enhanced release of EVs at a rate that was proportional to the measured sensitivity of the cells [50].
19 The increased production of EVs allowed more drug to be exported and thus the cells to be more
20 resistant [50]. The orientation of some drug transporters may also be reversed in the membrane of
21 EVs, which would allow these proteins to pump drugs into the vesicles rather than out, consistent
22 with a role in mediating expulsion of the drugs in EVs and thus in mediating resistance [51].
23 Sequestration of cytotoxic compounds by EVs is therefore another potential mechanism by which
24 tumors can gain resistance to therapy.

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

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

35 The application of proteomic technology to analyse the protein content of EVs can give insight into
36 the mechanisms by which EVs can cause resistance, and could also be used to identify biomarkers
37 for the status of a tumour. A range of techniques can be used to analyse the vesiculome, including
38 gel-based proteomics (in which proteins are separated on a gel and excised bands are analysed by
39 mass spectrometry (MS), for example) and gel-free approaches (where total proteins are
40 fragmented and analysed using techniques such as mass spectrometry) [53]. A comprehensive
41 proteomic analysis of multiple cell types suggests that EV protein content closely reflects the cellular
42 source of origin, suggesting that they offer a good indicator of cellular condition [54]. Exosomes
43 released by apoptosis-resistant acute myeloid leukemia (AML) cells contain differences in their
44 proteome, compared to those released by sensitive AML cells. Some of these proteins, which were
45 related to processes including apoptosis and splicing, could be transferred between cells [55].
46 Proteomic analysis of EVs from prostate cancer cells that were sensitive or resistant to taxane
47 revealed an upregulation of integrin β 4 and vinculin in the EVs from resistant cells [56]. Similarly,
48 another study showed that EVs from prostate cancer cells that were docetaxel resistance differed in
49 their protein content compared to those released by sensitive cells. Interestingly MDR1, MDR3,

1 Endophilin-A2 and PABP4 were up-regulated in EVs from resistant cells [57]. MDR1, MDR3 and
2 PABP4 were also upregulated in the sera of a small sample of three patients with resistant prostate
3 cancer [57], suggesting they could be used as a biomarker to predict treatment response. Proteomic
4 analysis of EVs could also predict likelihood of side effects of treatment; one study showed that a 12-
5 protein signature in serum EVs predicts the likelihood of chemotherapy-induced peripheral
6 neuropathy in breast cancer patients [58]. Proteomics can therefore give an important insight into
7 the mechanisms by which EVs could contribute to drug resistance.

8 **Transfer of EV protein can cause transfer of drug resistance**

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

32 **Transfer of EV RNA can cause transfer of drug resistance**

33 miRNA

34 EVs can carry a variety of coding and non-coding RNA cargo. miRNAs are short (19-25 nucleotides)
35 non-coding RNAs that primarily function by binding to the RNA-induced silencing complex (RISC) and
36 repressing gene expression via targeting of 3'UTRs of specific mRNAs [76, 77]. They can also function
37 as ligands to RNA-binding proteins such as Toll-like receptors, leading to the activation of signalling
38 pathways [78]. They have been implicated in stress response [79] and in cancer progression [76, 80,
39 81]. Importantly, their deregulation can promote drug resistance in tumors of different origins [82].
40 It is therefore not surprising that miRNAs transferred via EVs may play a role in mediating the
41 transfer of resistance from one cell to another

42 Transfer of miRNAs can occur between docetaxel-resistant MCF7 cells and their sensitive parental
43 line [83-85]. These miRNAs can cause downregulation of target genes in the recipient cells [83].
44 RNase treatment of the vesicles abrogates the transfer of resistance, suggesting RNA molecules are
45 important in the mediation of the effect [83]. Transfer of miR-21, miR-27a and miR-451 from a
46 chronic myeloid leukemia cell line to MCF7 cells led to increased resistance, possible via activation of
47 Akt signalling [66]. EVs from a cisplatin-resistant ovarian cancer cell line (CP70) can transfer
48 resistance to the sensitive parental line (A2780) via vesicular transfer of miR-214 and miR-21-3p,

1 which induce resistance by repressing PTEN [86] and NAV3 [87], respectively. miRNA-155 in EVs
2 from gemcitabine-resistant pancreatic cancer cells can induce resistance when taken up by sensitive
3 recipient cells; miR-155 induces this resistance by targeting the pro-apoptotic gene TP53INP1 [88].
4 Transfer of miR-96 in lung cancer cells (from H1299 to A549 cells) may cause resistance to cisplatin
5 that is mediated by targeting of the LIM-domain only protein 7 (LMO7) [89]. Another study with the
6 same pair of cells showed that transfer of miR-222 may be involved in mediating resistance to
7 adriamycin [90]. miR-221/222 can be transferred from tamoxifen resistant MCF7 to the sensitive
8 parental cells leading to acquisition of resistance, possibly via targeting of P27 and ER α [91]. Taken
9 together these studies show that transfer of extravesicular miRNA is one of the mechanism by which
10 resistance can spread between cells.

11 mRNA and long non-coding RNAs (lncRNAs)

12 In addition to carrying miRNAs it is known that EVs can contain many other species of RNA, including
13 coding mRNAs and lncRNAs. It is known that mRNAs can be transferred into recipient cells and
14 actively translated into functional protein [32]. In addition to transferring P-gp protein to recipient
15 cells, many resistant lines can produce EVs carrying *MDR1* (*ABCB1*) or *MRP1* (*ABCC1*) mRNA which
16 can be transferred into sensitive cells to induce resistance [61, 66, 69, 75, 85]. A recent study
17 highlighted the complexity of relationship between mRNAs and miRNAs delivered using leukaemia
18 cell line CCRF-CEM and its multidrug resistant derivatives VLB₁₀₀ and E₁₀₀₀ [92]. The authors showed
19 that *ABCB1* (also known as *MDR1*), *ABCC1* (also known as *MRP1*) and miR-326 can all be delivered in
20 EVs, but that expression of *ABCB1* dominates whilst miR-326 represses *ABCC1*. If *ABCB1* is knocked
21 down then miR-326 represses *ABCC1* less efficiently, suggesting a complex interplay between RNA
22 delivered in EVs.

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

36 The role of lipids in vesicular resistance-transfer

37 EVs are bound by a bilayer that contains a different balance of lipids compared to normal plasma
38 membrane. They are enriched in various lipids, including ceramide, sphingomyelin,
39 phosphatidylcholine, diacylglycerol and gangliosides [98, 99]. Interestingly, ceramide is required for
40 EV biogenesis and is required for the loading of some of the cargo in vesicles [100, 101]. Ceramide
41 has also been linked to drug resistance, possibly via P-gp activity and sequestration of drugs in EVs
42 [40, 100, 102, 103]. A recent study of the lipidomics of EVs released by PC9R cells (a non-small cell
43 lung cancer cell line resistant to Gefitinib) reveals a number of phospholipids that are over- or under-
44 represented compared to the EVs released by the sensitive parental line PC9 [104]. Another study
45 showed that artificial EV-like nanoparticles (composed of lipids similar to those found in EVs) could
46 induce activation of NF- κ B in MiaPaCa-2 cells (a pancreatic cell line) which then induces secretion of
47 the chemokine SDF-1 α [105]. This in turn interacts with the CXCR4 receptor on the surface of cancer
48 cells and induces resistance via Akt signalling [105]. These results suggest that lipid biosynthesis and
49 signalling may play a role in mediating resistance via transfer of EVs.

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

2 Thus far we have discussed the way in which EVs transferred from resistant cancer cells can induce
3 resistance when taken up by other, more drug-sensitive, cancer cells. However, tumor cells do not
4 normally grow in isolation. In their native context cancer cells live alongside a variety of other cell
5 types, which together are referred to as the tumor microenvironment [106]. Cross-talk between
6 cancer cells and stromal cells has emerged as an important factor in the progression of a tumor
7 [107]. A variety of stromal cells interact with cancer cells, including cancer associated fibroblasts
8 (CAFs), tumor associated macrophages and endothelial cells. Indeed, the tumor microenvironment is
9 now recognised as having at least as much complexity as any other organ. One of the methods by
10 which cancer and stromal cells communicate is via the exchange of EVs. This EV-mediated
11 communication, which occurs in a regulated fashion between epithelial cells and stromal cells, can
12 become corrupted during tumorigenesis and can promote tumor progression and resistance to
13 therapy [37, 108].

14 Numerous examples of EV transfer between stromal cells and cancer cells leading to resistance have
15 now been documented. MSC exosomes could increase resistance of gastric cancer cells to 5'FU
16 [109]. Treatment of colorectal cancer stem cells (either from patient xenografts or cell lines sorted
17 for CD133) showed increased resistance (to chemotherapeutic agents 5-fluorouracil and
18 oxaliplatin) when treated with EVs from CAFs [110]. Stromal cells can transfer galectin-3 via vesicles
19 to acute lymphoblastic leukaemia cells that induce endogenous production of further galectin-3 and
20 NF-kB activation which leads to drug resistance [111]. Bone marrow stromal cells (BMSCs) release
21 EVs that can induce resistance to bortezomib in multiple myeloma cells [112]. This may involve
22 activation of survival signalling pathways including JNK, p38, p53 and Akt [112]. RNA in exosomes
23 from stromal cells can induce resistance in breast cancer cell by triggering signalling via the pattern
24 recognition receptor RIG-I and via NOTCH3, leading to the activation of a STAT1-dependent antiviral
25 response [113].

26 A common theme in the acquisition of drug resistance via EV transfer within the tumor
27 microenvironment is the transfer of functional miRNAs [114]. For example, cancer associated
28 adipocytes (CAAs) and fibroblasts (CAFs) transfer vesicular miR-21 to ovarian cancer cells leading to
29 increased paclitaxel resistance via targeting of the apoptosome component APAF1 [115]. Treatment
30 of CAFs with gemcitabine leads to increased release of EVs with the capacity to induce resistance in
31 pancreatic cancer cells by transferring mRNA encoding the resistance factor Snail and also miR-146a
32 [116]. Mesenchymal stem cells primed by interaction with breast cancer cells release EVs with miR-
33 222/223 that induce quiescence in a proportion of the cancer cells and induce drug resistance [117].
34 Bone marrow mesenchymal cells taken from human donors were able to repress proliferation and
35 increase resistant bone marrow-metastatic cell line derived from MDA-MB-231 breast cancer cells
36 [118]. The mechanism involved transfer of miR-23b into the recipient cells which repressed the cell
37 cycle regulator MARCKS [118]. Interestingly, EV-mediated communication can occur in both
38 directions. For example, neuroblastoma cells release EVs with miR-21 which interact with the Toll-
39 like receptor in monocytes, leading to reciprocal release of EVs from monocytes carrying miR-155,
40 which in turn enter neuroblastoma cells and repress TERF1 to induce cisplatin resistance [119].
41 These results demonstrate that EV-mediated cross-talk within the tumor microenvironment is an
42 important mechanism by which resistance can be transferred to cells.

43 **Potential role of EV-mediated bystander effects**

44 Another interesting factor in drug resistance is the role of EVs during stress response which could
45 lead to bystander effects. The bystander effect (BE) is a phenomenon in which cells undergoing
46 stress can communicate with other cells to coordinate an intercellular response [120]. The BE was
47 originally described using radiation; when cells are exposed to ionizing radiation they release a
48 secreted signal which can induce DNA damage when placed onto naïve cells [121]. This effect has
49 also been observed following treatment of cells with other stress types, including cytotoxic

1 chemicals [122, 123] and heat stress [124]. We have recently shown that the BE is mediated by
2 release and uptake of EVs following irradiation[125], cytotoxic stress [127] or heat [128]. The
3 observation that BE is evolutionarily conserved implies a potential role in organismal fitness. One
4 potential benefit to the BE is that the bystander cells, although apparently stressed, are in fact more
5 robust in the face of future insults. In other words, EVs released from stressed cells can induce an
6 adaptive response in naïve recipient cells[127-130]. Applied to the cancer therapy setting one could
7 imagine a situation where in a cohort of tumor cells treatment with radio- or chemotherapy could
8 induce release of EVs into the extracellular space which could induce an adaptive response within
9 the population. Evidence of such a model does exist in the literature. For example, treatment of
10 A549 cells with EVs released by cisplatin-treated A549s increased resistance to cisplatin [131].
11 Treatment of pancreatic cells (MiaPaCa and Colo-357) with gemcitabine led to release of EVs with
12 capacity to induce resistance to gemcitabine [132]. This was partly mediated by transfer of miR-155
13 which represses DCK (a gemcitabine metabolising enzyme) and transfer of SOD2 and CAT which can
14 both detoxify reactive oxygen species [132]. Treatment of hepatocellular carcinoma induces
15 upregulation of lncRNAs in EVs that can cause resistance when placed onto naïve cells [96, 97]. EV-
16 mediated communication during chemotherapy could therefore also be a mechanism by which
17 resistance arises within the tumor mass.

18

19 **Perspective and future directions**

20 The body of work reviewed here provides a compelling case for the role of EVs in mediating drug
21 resistance. However, many questions remain unanswered. The full range of mechanisms by which
22 EVs induce resistance has certainly not been fully elucidated, so further work is necessary. For
23 example, in some studies a role for activation of signalling pathways such as HGF/c-Met/Akt can be
24 established [133], but not the precise mechanism by which they become activated. In some cases
25 there is also contradictory evidence; knockdown of *MDR1 (ABCB1)* in donor cells does not always
26 have an effect on the transfer of resistance, suggesting that either the role of *MDR1/P-gp (ABCB1)*
27 transfer is complex, or that this particular transfer represents one of multiple mechanisms by which
28 induction of resistance can be achieved by EVs [109]. Other studies suggest that transfer of EVs does
29 not always lead to increased resistance. For example, in one study using androgen independent
30 prostate cancer cell line DU-145, transfer of EVs from resistant cancer cells to their parental line did
31 not induce resistance [57], whereas other studies showed that DU-145 EVs induce resistance [42,
32 61]. These conflicting results may be due to subtle differences in methodology such as drug
33 concentration used, incubation time or thresholds set, the type of drug resistance being tested, or
34 could suggest that EVs have context-dependent effects. For this reason it is important that all results
35 of EV-transfer experiments are published and that 'negative' results are not excluded from the
36 literature.

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

47 **Potential for therapeutic and prognostic applications**

48 Better understanding of the way EVs mediate drug resistance could lead to novel combination
49 treatments that are more effective. EVs appear to mediate bystander effects that occur following

1 exposure of cells to radiation or cytotoxic drugs; given that these effects include an adaptive
2 response it should be possible to sensitise cells to treatment if EV transfer is inhibited. Indeed,
3 treatment of cells with heparin (an EV uptake-inhibitor) can sensitise cells to cisplatin [137], and our
4 data suggest that this may be due to the inhibition of EV uptake [127].

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

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

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

42 Final remarks

43 It has become very clear in the last few years that EVs are an important part of the dialogue that
44 occurs between different cells [25]. Tumor cells also release EVs, allowing them to transfer
45 macromolecules to other cancer cells or stromal cells in the tumor microenvironment. This transfer
46 can have a range of phenotypic effects, but ultimately many of these support the growth of the
47 tumor. Importantly, it can lead to the transfer of resistance from one tumor cell to another. One can
48 envisage how this would allow resistance across a population of cells in a tumor mass to rise, as
49 resistance could be acquired in a pocket of the heterogeneous mass and then spread to other cells

1 via the release of EVs. This, then raises the possibility that drug resistance may be modulated by
2 locally inhibiting EV release or uptake. This offers an exciting new insight into the biology of cancer,
3 and offers potential therapeutic targets for tackling the acquisition of therapeutic resistance. Further
4 work must be performed, particularly *in vivo*, to better elucidate the mechanisms by which transfer
5 of EVs can induce resistance.

6 **Acknowledgements**

7 We thank the Cancer and Polio Research Fund and Oxford Brookes University for funding. We
8 apologise to all the authors whose excellent work could not be included in this review due to space
9 constraints.

10 The authors have declared no conflict of interest

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15 **Figure Legends**

16

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

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

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1 **Tables**

2 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]
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