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Review

Extracellular Vesicles: Subcellular Organelles With the Potential to Spread Cancer Resistance*

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Abstract. *Although modern anticancer drugs have made great progress in disease treatment, the occurrence of drug resistance often leads to treatment failure. Understanding the molecular basis of resistance mechanisms is important to determine prognosis and develop strategies for circumvention. In this context, subcellular vesicles released by cancer cells have been identified to mediate cellular resistance by various mechanisms. Such extracellular vesicles (EVs) can be subdivided into exosomes and ectosomes based on their size, cargo, and mechanism of formation. The unveiling of EV-targeted treatment options depends on a sound knowledge on EV biology including biogenesis, release, targeting to recipient cells, and uptake. In this review, we focus on EVs as mediators of cancer drug resistance with a particular emphasis on the distinction of exosomes and ectosomes.*

With the development of new therapeutic approaches, such as targeting signaling pathways by inhibitors and specific antibodies, modern cancer treatment has achieved considerably higher remission rates and better treatment tolerance for patients than a decade ago (1). Nevertheless, the development of drug resistance with subsequent treatment failure remains a major problem and we are still learning the mechanisms underlying cancer cell resistance (2). In this context, subcellular vesicles released by cancer cells are newly identified candidates believed to mediate resistance. Several mechanisms, such as drug export/sequestration, horizontal transfer of membrane-bound proteins, transfer of genetic information, and neutralization of antibody-based drugs, have been identified so far. Extracellular vesicles (EVs) differ in size and mechanism of formation, as well as in the cargo they carry (3). These differences may explain why certain subpopulations of EVs are linked to distinct drug resistance mechanisms. Herein, we give an overview of the current knowledge regarding EVs as mediators of cancer drug resistance.

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Cellular Resistance Mechanisms

Based on in-depth molecular exploration of cancer cell biology, new strategies of cancer treatment have been developed in the past decade. Use of checkpoint inhibitors and immunotherapeutic approaches in particular, have resulted in considerably higher remission rates and enabled treatment of formerly resistant cancer (4). The downside of these treatment principles is that the more specific a drug is in blocking a particular pathway, the more likely is the development of a cellular resistance mechanism during therapy that circumvents this pathway blockage.

In principle, there are two distinct resistance mechanisms, namely inherent and acquired. Whereas the first develops during cancer genesis, acquired drug resistance arises under the pressure of treatment with specific anticancer drugs. Besides cancer cell alterations, a number of factors such as pharmacokinetic mechanisms, under-dosage based on clinical considerations, and epigenetic changes may contribute to the clinical phenomenon of drug resistance, and subsequently to treatment failure (5, 6). In addition, especially in modern immunotherapeutic approaches, the tumor microenvironment as a crucial element in treatment success has to be taken into account (7, 8).

Biogenesis of EVs: Exosomes and Ectosomes

EVs are found in subsets that differ in size, cargo, and mechanism of formation. Exosomes are small membrane vesicles (30-100 nm) that form intracellularly within multi-vesicular bodies (MVB), and are released after fusion with the plasma membrane. Larger ectosomes (50-1000 nm), on the other hand, directly arise by outward budding and fission of the plasma membrane (9).

Mechanistically, the machinery of the endosomal-sorting complex required for transport (ESCRT) is reportedly involved in exosome generation. This system acts as a driver of membrane shaping and scission, and hence is involved in the formation of multi vesicular bodies and intraluminal vesicles (10). Inactivation of the ESCRT affects the efficiency of exosome secretion as well as the composition of the secreted vesicles (11). In addition, exosomes can also be formed in an ESCRT-independent manner involving the generation of ceramide (12, 13), or proteins of the tetraspanin family (such as CD63, CD81, CD82, CD9), which participate in imposing a spontaneous negative curvature on the membranes and in endosomal sorting, respectively (14-17). Since the endosomal-sorting machinery is recognized as the major regulator of exosome composition, agents or activities affecting these processes should be considered when investigating exosome biogenesis as well as manipulation.

The biogenesis of ectosomes, on the other hand, requires some rearrangements within the plasma membrane. Dependent on the intracellular Ca^{+2} concentration, activation of enzymatic machinery such as translocases (flippases and floppases), scramblases, and calpain facilitate migration of phosphatidylserine, normally confined to the inner cytoplasmic membrane leaflet, across the lipid bilayer to the cell surface. This leads to bending of the membrane, restructuring of the cytoskeleton, and finally results in membrane budding (18, 19). However, ectosome formation can occur even when membrane lipid asymmetry is maintained (20, 21). In this regard, it has been shown that the presence of cholesterol and regulators of cytoskeletal elements are also essential for ectosome generation (22-24).

Notably, although the generation of exosomes and ectosomes occurs at distinct sites within a cell, both EV populations also share intracellular mechanisms and sorting machinery in the process of their biogenesis, which makes it difficult to distinguish them in some cases (25).

Release of EVs. Especially under inflammatory conditions, EVs can be released into the extracellular space by all cells. Initially, EV secretion was seen as a cellular waste-disposal process (26) but today it is generally accepted as a directed and highly regulated process (3, 25, 27). The release of exosomes is a complex procedure that involves the sorting of cargoes into MVBs and further into intraluminal vesicles. Moreover, MVBs need to be prevented from degradation, targeted to the plasma membrane, and primed for secretion (9). All these steps probably require additional regulatory checkpoints, which likely results in a time difference between the formation and the release of exosomes compared to ectosomes. The release of ectosomes, on the other hand, seems to be the direct consequence of membrane budding and fission. This process requires the interaction of actin and myosin with subsequent ATP-dependent contraction (24). Of note, ESCRT-dependent release involving tumor susceptibility gene 101 (*TSG101*) and vacuolar protein sorting 4 (VPS4) ATPase has been reported not only for exosomes but also for ectosomes (28). Furthermore, it is known that an increased Ca^{2+} concentration boosts the release of ectosomes from cells (29).

Targeting to recipient cells. Once released into the extracellular space, EV-mediated intercellular communication requires membrane docking, activation of surface receptors and signaling, endocytosis of EVs, or fusion with the membrane of the recipient cell. Specificity for the recipient cell is determined by definite interactions between surface structures of EVs and receptors at the plasma membrane of the cell. Examples of such structures are tetraspanins (30), integrins (31), lipids (32), heparan sulfate proteoglycans (33, 34), and extracellular matrix components (35, 36).

Cellular uptake of EVs. To transfer a specific piece of information, EVs interact with, or can be taken up by recipient cells *via* multiple mechanisms such as surface binding and subsequent fusion, macropinocytosis, phagocytosis, and different endocytic mechanisms that are either clathrin-dependent or function *via* clathrin-independent pathways such as caveolae or lipid rafts (9).

The specific composition of EVs can be directly liked to their fate. For example, amyloid precursor protein containing exosomes from neuroblastoma cells were specifically endocytosed by neurons (37). Furthermore, on throphoblast-derived exosomes, syncytin 1 acts as an ‘eat me signal’, promoting their uptake (38). Notably, the dynamics of interaction is also dependent on membrane structures on the recipient cell. It has been reported that filopodia on target cells mediate the transfer of EVs towards endocytic hot spots (39). In particular, lipid rafts on the target cell have been shown to contribute to EV internalization, as their disruption resulted in reduced uptake of EVs (40). Once taken up, EVs are collected into MVBs following the pathway of endocytosis, which leads to vesicle degradation upon fusion with lysosomes in most cases (41). Interestingly, through back fusion with the MVB membrane, internalized vesicles can escape from digestion, and thus can release their cargo into the cytoplasm of the recipient cell (42).

Signal transmission by EVs. Docking of EVs at the plasma membrane can result in the activation of surface receptors, signaling, and the onset of functional responses in target cells. For example, antigen-presenting EVs have been shown to be potent inducers of specific antigenic responses in T-cells (43, 44). Furthermore, fibronectin detected on tumor-derived EVs promoted their anchorage-independent growth after binding to integrin on non-transformed fibroblasts (45). Besides receptor activation at the surface, internalized EVs can activate responses through the delivery of their cargo. Similarly to antigens, protein cargo can be processed in the endocytic compartment and used for antigen presentation involving EVs in the process of immune regulation (31, 46). When EVs directly fuse with the plasma membrane of the target cell, they release their intraluminal content, such as miRNA or mRNA, into the cytoplasm and can mediate gene expression (47, 48). Upon membrane fusion, EVs also transfer membrane lipids (such as eicosanoids and fatty acids) and proteins [such as epidermal growth factor receptor variant III (EGFRvIII)], thereby contributing to the regulation of bioactive lipid species (49) and to a horizontal propagation of oncogenes and their associated transforming phenotype, respectively (18).

Nomenclature of EVs

To date (May 2019), consensus has not yet emerged on specific markers of EV subtypes. Therefore, the International Society for Extracellular Vesicles has suggested an EV nomenclature that refers to physical characteristics, biochemical composition, or descriptions of conditions or cellular origin (50). However, EV subtypes have overlapping sizes, which impairs methods for EV isolation based solely on this parameter. Reports on biological effects of EV subtypes have to be critically viewed in light of the method used for purification. For example, an immediate ultracentrifugation of body fluids or cell culture supernatants at 100,000 × *g* would pelletize not only small exosomes but also larger ectosomes, resulting in a mixture of both populations so that observed biological effects cannot be clearly assigned to one or the other set of EVs. Due to the difficulties of standardized EV subgroup distinction, we refer to exosomes, ectosomes, microparticles/microvesicles and oncosomes as EVs. Apoptotic bodies, which are a special form of EV released by dying cells, are not considered in this review.

EV-linked Drug Resistance Mechanisms

Since EVs comprise all biomolecular categories including proteins, lipids and nucleic acids (3), the development of drug resistance *via* EVs is linked to mechanisms involving such cargo.

EV-mediated drug export or sequestration. The EV-mediated export or sequestration of cytotoxic drugs reduces their effective concentration in target cells. The major cellular mechanism using this principle is the overexpression of membrane transporters that reduce intracellular drug levels to sublethal concentrations (51). As early as 2003, a positive correlation between genes involved in EV shedding and drug resistance in cancer cell lines was described (52). In the same study, it was found that breast cancer cells were able to export the chemotherapeutic agent doxorubicin into the extracellular medium *via* vesicle formation (52). Similarly, resistant ovarian carcinoma cells (53) as well as melanoma cells (54) were able to deposit cisplatin within EVs. On the molecular level, EVs from resistant cells overexpressed proteins involved in detoxification of cisplatin such as multidrug resistance-associated protein 2 (MRP2), as well as ATP7A and ATP7B transporter proteins (53). Furthermore, in a breast cancer model, the administration of mitoxantrone led to rapid sequestration of the drug in EV-like structures at the plasma membrane, and this was linked to the overexpression of the multidrug efflux transporter ABCG2 [also known as breast cancer resistance protein (BCRP)] in these cells (55).

EV-mediated horizontal transfer of membrane-bound proteins. As already mentioned, tumor cells can achieve resistance through the delivery of membrane-bound drug efflux pumps to sensitive cells. Among such transporters, multi-drug resistance-associated P-glycoprotein (P-gp, MDR-1 or ABCB1) is one of the well-studied ones. Not only were resistant variants of cell lines found to express ABCB1, but EVs from the resistant variants also carried ABCB1 and, therefore, may have the ability to confer resistance to docetaxel at least in part by the EV-mediated transfer of ABCB1 (56). In addition, the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway may contribute to the regulation of the subcellular localization of ABCG2 (57) and ABCA3 (58). It has been postulated that the PI3K-AKT signaling pathway may also play a role in the exclusive sorting of ABCG2 to the membrane of EVs in multidrug resistance in breast cancer cells (MCF-7/MR) (56). The unique localization of ABCG2 allowed for efficient pumping and hence concentration of multiple cytotoxic agents of distinct structure and mode of action, as well as non-toxic compounds including riboflavin (59), from the cytoplasm to the lumen of EVs. Importantly, blockade of the AKT signaling axis markedly increased the cytoplasmic localization of ABCG2, resulting in reduced drug accumulation within EVs and subsequent reversal of multi drug resistance (56).

Of note, similar transfer mechanisms have been reported for models of ovarian cancer (60), leukemia (61), and osteosarcoma (62).

EV-mediated transfer of genetic information relevant for resistance. Surrounded by a membrane, specific bioactive cargoes, such as proteins and nucleic acids, are conserved in EVs and thus, protected from degradation. In recipient cells, such cargoes may alter cell cycle control and apoptotic programs favoring tumor cell survival and growth. For instance, different microRNAs (miR) have been found in EVs from neuroblastoma cell lines. Among those, *miR-21* was the top represented. When EVs were co-cultured with monocytes, cells acquired *miR-21* very rapidly. Monocytes that acquired *miR-21* transcribed oncomiR-155 and subsequently released oncomiR-155-containing EVs that were internalized by neuroblastoma cells, resulting in an increased resistance to the drug cisplatin (63). The horizontal transfer of EVs that contain anticancer drug resistance-promoting miRs has been shown for a variety of cancer types [e.g. lung (64, 65), breast (66, 67), pancreatic (68), colonic (69), prostatic (70), melanoma (71), glioblastoma (72), and leukemia (73, 74)]. Of note, an enhanced secretion of tumor-suppressor miRs *via* EVs upon exposure to chemotherapeutic agents can also serve as disposal mechanism, leading to reduced drug sensitivity of tumor cells (69).

EV-mediated neutralization of antibody-based drugs. Since EVs share features of antigenicity with the cells of their origin, the presence of antigens targeted by immunotherapy acts as a sink for antibody-based drugs. In B-cell lymphoma cell lines and primary lymphoma cell preparations, the expression of CD20 on B-cells was indeed mirrored by EVs. CD20 on EVs bound the therapeutic antibody to CD20 rituximab and effectively depleted the soluble antibody from antibody suspensions. Moreover, in patients who had received the antibody for therapeutic purposes, approximately half of the plasma rituximab was fixed to EVs 3 h after the end of the infusion (58). In addition, in different breast cancer models, human epidermal growth factor receptor 2^+ ($HER2^+$) EVs were found to play a role in modulating resistance to the HER2 antibody trastuzumab. Either secreted by $HER2^+$ tumor cells *in vitro* or present in the serum of patients, EVs were found to bind to trastuzumab, and block its activity *in vitro* (75). More recently, the immune checkpoint ligand programmed cell death 1 ligand 1 (PD-L1) was detected on EVs from patients with melanoma (76). By capturing immunotherapeutic antibodies to PD-L1, EVs drive the drug away from the tumor and thereby break down T-cell function and antitumor immunity.

Possible Therapeutic Options Targeting EV-mediated Resistance Mechanisms

Using EV-inhibiting agents may sensitize cancer cells to chemotherapeutic agents and reduce cancer growth in patients. To discuss possible therapeutic options, it would be desirable to distinguish between exosome and ectosome-related resistance mechanisms and to consider the levels of EV generation, release and interaction with the recipient cell. As we are still at the beginning of our understanding of EV biology, most of the targeting levels are still hypothetical.

Intracellular cargo. Tetraspanins affect the intracellular routing of cargos such as integrins towards MVBs, suggesting that functional impairment would affect different steps of exosome generation (77).

EV release. It was shown that cannabidiol, a phytocannabinoid derived from *Cannabis sativa*, acts as a potent inhibitor of EV release *in vitro* (78). Furthermore, chloramidine and bisindolylmaleimide-I have been shown to inhibit EV release without affecting cell viability. Apoptosis mediated by the chemotherapy drug 5-fluorouracil (5-FU) was significantly enhanced in the presence of both inhibitors (79).

EV docking. We still know too little about the structural prerequisites for EVs to find and dock on recipient cells. It would be interesting to develop targeted drugs that specifically inhibit the binding of EVs.

EV uptake. It has been shown that depletion of cholesterol disrupts lipid rafts on the surface of the recipient cell and considerably reduces the uptake of EVs (40).

Therapeutic removal of EVs. Tumor-derived EVs are involved in tolerating and modulating their environment, promoting metastasis and angiogenesis, immunosuppression and drug resistance (80). Removal of such mediators from patients could improve anticancer therapy. As early as 1989, when EVs had not even been identified, removal of low-molecular-weight proteins reportedly resulted in tumor shrinkage (81).

The recently developed hemofiltration technology of Adaptive Dialysis-like Affinity Platform Technology (ADAPT™; Aethlon Medical, Inc., San Diego, California) separates blood components <200 nm. With target antigens interacting with immobilized agents of the affinity matrix, such as monoclonal antibodies, lectins and aptamers, disease-related components can be removed from the entire circulatory system without loss of essential blood components (82). By capturing tumor-secreted exosomes that suppress the immune system and thus contribute to drug resistance in cancer, the device enables immune-based therapy with improved clinical outcome without the danger of increasing drug toxicity or interaction risks.

Summary and Concluding Remarks

For more than a decade, the involvement of EVs in facilitating anticancer drug resistance *via* several different mechanisms has

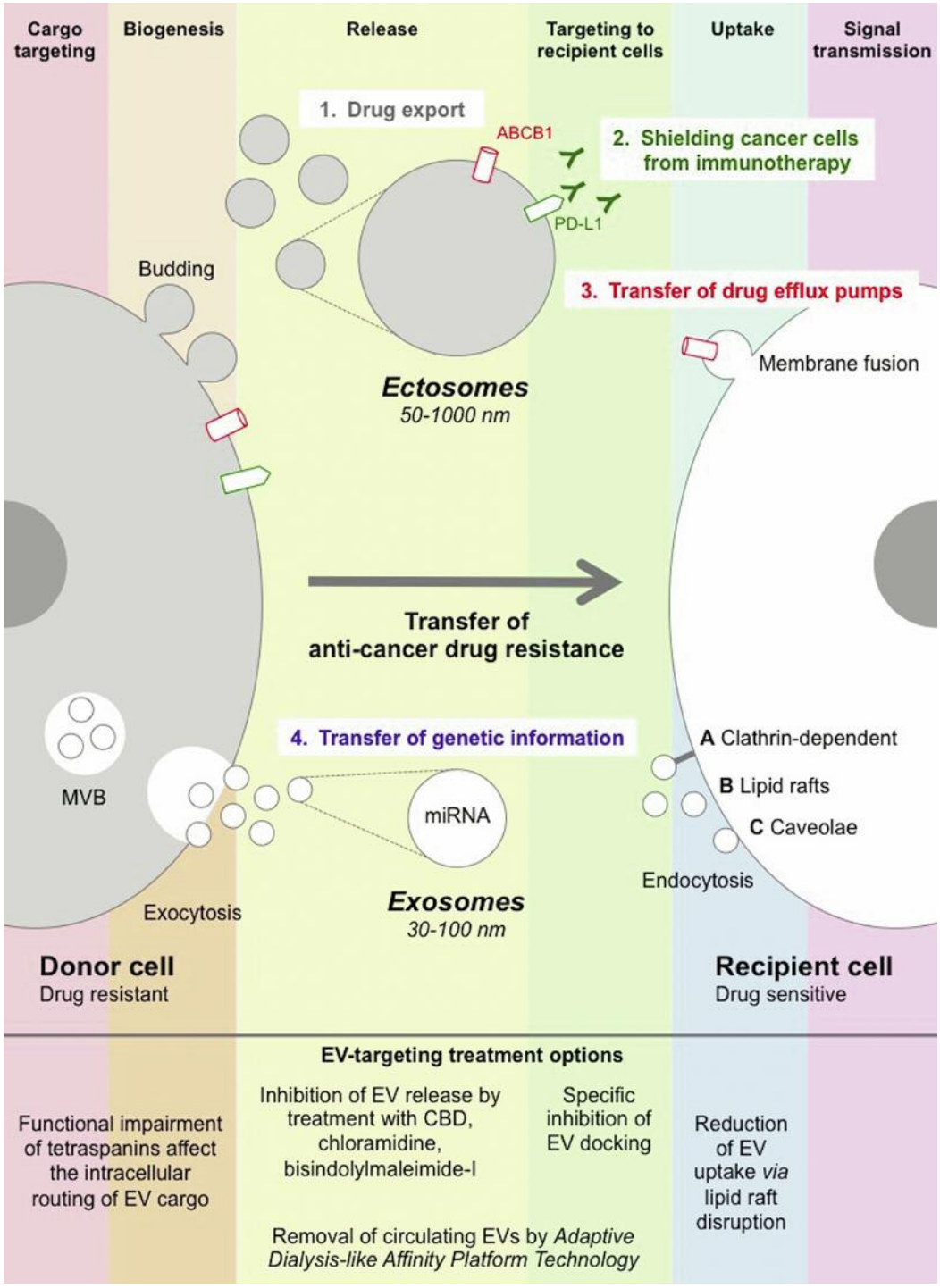

Figure 1. *The role of extracellular vesicles (EVs) in spreading anticancer drug resistance. EVs comprise a heterogeneous population of membrane vesicles of various origins. Being either exocytosed from intracellular multivesicular body (MVBs) storage upon fusion with the plasma membrane, or directly budded from the cell surface, exosomes differ from ectosomes. Both populations are secreted under physiological as well as pathophysiological conditions, such as cancer genesis and the development of anticancer drug resistance. Interestingly, it has been revealed that EVs are involved not only in the desensitization of cancer cells upon treatment with chemotherapeutic drugs (i. Drug export) or cancer immunotherapeutic agents (ii. Shielding cancer cells from cancer immunotherapy), but also in the spread of anticancer drug resistance from resistant to sensitive cancer cells through numerous mechanisms (e.g. iii. Transfer of drug efflux pumps; iv. Transfer of genetic information). Discovery of EV-targeted treatment options strongly depends on a sound knowledge about EV biology including biogenesis, release, targeting to recipient cells, and uptake. ABCB1: ATP-binding cassette transporter, sub-family B member 1 (also known as P-glycoprotein); CBD: cannabidiol; miRNA: microRNA; PD-L1: programmed cell death 1 ligand 1.*

been demonstrated (Figure 1). However, since specific markers for a clear distinction of EV subtypes have not yet been defined, mechanisms of anticancer drug resistance cannot be linked to any subtype. According to a worldwide survey by the International Society for Extracellular Vesicles in 2016, sequential ultracentrifugation was the most commonly used method for primary EV separation and concentration (83). Others and we have used this method, with small adaptations, to successfully separate larger from smaller EVs, showing differences in content and functionality of those subpopulations (84-87). In particular, Crescitelli and colleagues have shown that such EV subpopulations differ in their RNA content. While smaller RNAs without prominent ribosomal RNA peaks were detectable in small vesicles (exosomes), larger vesicles (ectosomes) contained little or no RNA, at least in an *in vitro* system (84). Furthermore, we have shown that larger vesicles (ectosomes) carry higher amounts of membrane-bound tissue factor, a potent activator of the extrinsic coagulation cascade, as compared to small vesicles (exosomes) (85). In light of the differences in EV biogenesis, either from the inside of a cell or budding outward from the plasma membrane, these findings suggest the following hypotheses: miRNA-containing exosomes of small size are the drivers of resistance development *via* exchange of genetic information; and shedding of larger ectosomes facilitates detoxification of drug-loaded cancer cells and the horizontal transfer of membrane-bound proteins. For the future, it will be of major importance to re-evaluate previous findings on the biological effects of EV subpopulations based on a standardized protocol of EV isolation and distinction. This is especially important for the newer therapeutic approaches that involve selective inhibition of specific signaling pathways.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this review.

Authors' Contributions

All Authors contributed significantly to writing and correcting the article.

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