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Exosomal delivery of cannabinoids against cancer

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ABSTRACT

Exosomes are extracellular vesicles (EVs) originating from endosomes that play a role in cellular communication. These vesicles which mimic the parental cells that release them are promising candidates for targeted drug delivery and therapeutic applications against cancer because of their favorable biocompatibility, specific targeting, low toxicity, and immunogenicity. Currently, Delta-9-tetrahydrocannabinol (THC), cannabidiol (CBD) and other cannabinoids (e.g., CBG, THCV, CBC), are being explored for their anticancer and anti-proliferative properties. Several mechanisms, including cell cycle arrest, proliferation inhibition, activation of autophagy and apoptosis, inhibition of adhesion, metastasis, and angiogenesis have been proposed for their anticancer activity. EVs could be engineered as cannabinoid delivery systems for tumor-specificity leading to superior anticancer effects. This review discusses current techniques for EV isolation from various sources, characterization and strategies to load them with cannabinoids. More extensively, we culminate information available on different sources of EVs that have anticancer activity, mechanism of action of cannabinoids against various wild type and resistant tumors and role of CBD in histone modifications and cancer epigenetics. We have also enumerated the role of EVs containing cannabinoids against various tumors and in chemotherapy induced neuropathic pain.

1. Introduction

From the times of biblical plague to recent covid-19 pandemic, cancer has been a constant ailment and is responsible for approximately 15% deaths becoming the second-leading cause of death globally in accordance with American Cancer Society. In 2022, United States alone accounts for nearly 1.9 million new cancer cases with over 0.6 million cancer deaths [1,2]. The global burden is 10–15 times more than the United States' numbers according to Globocan's latest report [2]. Enormous amounts of funding and efforts have been invested in cancer research in the past semi-centennial. Current cancer therapies still lack high clinical success because of factors such as indistinguishable nature of cancer cells from healthy cells, side effects of conventional chemotherapy, drug resistance and non-specific targeting (see Tables 1 and 2).

Exosomes are extracellular vesicles (EVs), which are discharged into the extracellular microenvironment and carry cargoes which are reflective of the biogenesis pathways they underwent for their development. Briefly, they are created by the process of inward budding in early endosomes to form multivesicular bodies with an average size of 100 nm [3]. They operate as mediators of intercellular communication through direct cell-to-cell contact, or the transfer of chemicals released by one cell to another [4]. EVs have recently attracted huge scientific attention as a viable drug delivery technology that can overcome the drawbacks of synthetic nanoparticles.

Cannabinoids are the terpenophenolic compounds isolated from the strains of plant Cannabis (sativa, indica or hybrid) and have been used for centuries for their recreational as well as their therapeutic efficacy [5]. While the cannabis plants consist of a range of phytocannabinoids: tetrahydrocannabinol (THC), cannabidiol (CBD), tetrahydrocannabivarin (THCV) and cannabivarin (CBV) are amongst the most abundant and majorly bioactive cannabinoids. The cannabinoids are broadly classified as: phytocannabinoids, endocannabinoids and synthetic cannabinoids. The cannabinoids have been understood to show their biological effects by mediating the cannabinoid receptors CB1 and CB2 and via transient receptor potential vanilloid type 1 (TRPV1). However, recent studies have shown the role of 5HT1A receptors also in neurological disorders like epilepsy [6]. Further other mechanisms of cannabinoids are still being investigated. The medical use of cannabinoids has progressed from disorders of gastrointestinal tract, genitourinary tract, and infectious diseases to rather complex conditions of neurological disorders, pain, and cancer. The therapeutic role of cannabinoids has been extensively studied for the past few decades in a variety of cancers.

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Abbreviations

CBD	Cannabidiol
THC	Tetrahydrocannabinol
THCV	Tetrahydrocannabivarin
EVs	Extracellular vesicles
TRPV1	Transient receptor potential vanilloid type 1
TRM8	Transient receptor potential cation channel subfamily M (melastatin) member 8
CB1 CB2	Cannabinoid receptors 1 & 2
DOX	Doxorubicin
PTX	Paclitaxel
5-FU	5-Fluorouracil
ROS	Reactive oxygen species
EMT	Epithelial to mesenchymal transition
TME	Tumor microenvironment
NK	Natural killer
hUCMSC	Human umbilical cord mesenchymal stem cells
HEK 293	Human embryonic kidney 293
RT-PCR	Real time polymerase chain reaction
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand;
FAAH	Fatty acid amide hydrolase
MAGL	Monoacylglycerol lipase
HSP	Heat shock protein
HNSCC	Head and neck squamous cell carcinoma
ICAM-1	Intercellular adhesion molecule-1
PARP-1	Poly ADP-ribose polymerase 1
NSCLC	Non-small cell lung cancer
EGFR	Epidermal growth factor receptor
mTOR	Mammalian target of rapamycin
PI-3K	Phosphoinositide 3-kinase
MVBs	Multi-vesicular bodies
PD-L1	Programmed death-ligand 1
ILVs	Intra-luminal vesicles
ESCRT	Endosomal sorting complexes required for transport
MHC	Major histocompatibility complex
TEM	Transmission electron microscope
SEM	Scanning electron microscope
AFM	Atomic force microscopy
NTA	Nanoparticle tracking analysis
TNF	Tumor necrosis factor
DNMT	DNA methyltransferase
TDE	Tumor derived EVs
TET-2	Tet Methylcytosine dioxygenase 2
NSD-2	Nuclear receptor binding SET domain protein 2
MDA	Malondialdehyde
PIPN	Paclitaxel-induced peripheral neuropathy

A significant advancement in the use of cannabinoids in cancer came in the early 1970s, where cannabinoids were shown to demonstrate targeting and inhibiting tumor growth in various in-vitro models [7]. Subsequently, several preclinical studies suggested the anticancer effects of naturally occurring, synthetic and endocannabinoids in various in-vivo models of lung carcinoma, lymphomas, breast cancer, gliomas, prostate carcinomas, and neuroblastomas. The cannabinoids impart their anticancer effect by affecting the cell cycle, cell proliferation and apoptosis in tumor cells like any typical anticancer drug and also modulate multiple cancer related pathways such as AMPK, PPARϒ, mTOR, PKB, CAMKK-β and HIF-1α [8]. Cannabinoids have been critically evaluated in recent years for their possible anticancer properties and symptomatic pain relief in cancer patients. However, given that cannabis have the potential to impair cognitive functioning various reports have also highlighted the significance of safety requirements when using these substances. Further, poor pharmacokinetics with cannabinoids suggests the need of delivery systems to enhance their absorption. Hence, there is a need for better targeting, biocompatible and stable drug delivery systems, and exosomes (EVs) have displayed these features in recent years. Further, EVs receive bioactive components from their parent cells that may have therapeutic use in cancer. These principles and hypothesis are currently being investigated by various researchers.

Multiple reviews have recently been published to describe the biological functions and therapeutic potential of EVs and their payloads but a systematic overview of EVs and their cargo along with the role of cannabinoids encapsulation is missing in the literature. This review is unique because here, we have introduced the fundamental ideas for EVs (EV biogenesis, contents, and functions), exosomal cargo loading strategies with cannabinoids, characterization, and applications of these delivery systems in cancer and related diseases.

2. Major and minor cannabinoids

Cannabinoids, on the basis of their source of production are broadly classified into three categories as: phytocannabinoids, endocannabinoids and synthetic cannabinoids. They exert multiple effects in human body by modulating multiple cellular pathways via their interaction with the cannabinoid receptors (CB1 and CB2). Cannabinoid receptors along with other receptors of endocannabinoid system (ECS), G-protein receptors: GPR55, GPR35, GPR18, GPR119, GPR12; nuclear receptors: PPARϒ; and ligand gated channels: TRPV1, TRPV2, TRM8. Endocannabinoids (Anandamide, Arachdonoylglycerol) and synthesis/degradation enzymes (FAAH, MAGL) comprises the ECS. Phytocannabinoids are the compounds derived naturally from the plant *Cannabis sativa* or other strains of cannabis. They are a family of over 100 naturally occurring chemical compounds. They can be further divided into major and minor cannabinoids depending on the amount of their concentration in the plant. In general, THC (Delta-9 and Delta-8) and CBD are considered as the major cannabinoids, while, cannabinol (CBN), cannabigerol (CBG), cannabidiolic acid (CBDA), cannabidivarin (CBDV), cannabichromevarin (CBCV), cannabinolic acid (CBNA), tetrahydrocannabivarin (THCV), cannabichromene (CBC), cannabigerovarin (CBGV), tetrahydrocannabinolic acid (THCA) and others are regarded as minor cannabinoids [9]. Endocannabinoids are the endogenously derived ligands of the cannabinoid receptors which regulates and modulates number of physiological and pathological conditions [10].

Synthetic cannabinoids are the compounds developed synthetically to explore the ECS with increased therapeutic potential [11]. Currently FDA has approved different cannabis-derived drug products: Epidiolex (cannabidiol) and three synthetic cannabis-related drug products: Marinol (dronabinol), Syndros (dronabinol), and Cesamet (nabilone). Dronabinol (marketed as Marinol) is a synthetic form of delta-9-tetrahydrocannabinol (Δ⁹-THC), the primary psychoactive component of cannabis. Syndros is a liquid version of dronabinol, which was originally FDA-approved in 1985 in capsule form is marketed by AbbVie under the brand name Marinol. Nabilone is a synthetic Δ^9-THC analog. Although, similar in effects, nabilone is more potent than Δ^9-THC [12].

3. Role of cannabinoids in cancer

Among the family of heterogeneous cannabinoids, Δ^9-THC exhibits high psychoactive and abuse potential along with possessing robust anticancer activity. Most of the data available in the literature is with

Table 1
Studies listing different mechanisms of action of CBD in cancer treatment.

Type of Cancer	CBD's Mechanism of Action
Breast cancer citations	
Harpe et al., 2022 [32]	Activates TRPV1 channels and induces ER stress with increased Ca^{2+}influx, to elevate ROS and disrupt protein folding ultimately leading to decreased cell proliferation and increased apoptosis.
D'Aloia et al., 2022 [33]	Induces autophagy, apoptosis and bubbling cell death.
Jee Jo et al., 2021 [34]	Reduces proliferation, inhibits metastasis and stem cell like properties with marked decrease in the HIF-1α expression levels.
Morales et, 2020 [24]	Induces adherens junctions, upregulates β-catenin and E-cadherin expression levels and it also decreases the expression of IL-1β pathway proteins.
Schoeman et al., 2020 [27]	In combination with other cannabinoids, induces G2 cell cycle arrest and apoptosis.
Shrivastava et al., 2011 [23]	Role of receptor dependent and independent mechanisms and also apoptosis and autophagy as the main mechanisms for cell cytotoxicity.
McAllister et al., 2007 [31]	Downregulates Id-1 expression, an inhibitor of basic helix-loop-helix transcription factors (regulator of the fatal metastatic potential of the breast cancer cells).
Lung cancer citations	
Misri et al., 2022 [35]	Inhibits the growth and induces apoptosis in cisplatin resistant non-small lung cancer cells. It also reduces the tumor progression in mouse xenograft model.
Hamad et al., 2021 [36]	Induced cell death by activating caspase3/7 and increasing the expression of pro-apoptotic genes.
Milian et al., 2020 [13]	Inhibits cell proliferation and reduces EGFR expression in lung cancer cells. When used in combination with THC shows reversal epithelial to mesenchymal transition.
Ramer et, 2012 [30]	Role of intracellular adhesion molecule-1 (ICMA-1) in the increased levels of tissue inhibition of matrix metalloproteinases-1 (TIMP-1).
Haustein et al., 2014 [37]	increased susceptibility of the lung cancer cells to adhere to lymphokine-activated killer (LAK) cells and cell death induced by them by upregulating ICAM-1.
Gastric Cancer Citations	
Jeong et al, 2019 [38]	Targeted Smac induced downregulation of X-linked inhibitor of apoptosis (X-IAP) which triggers ER stress and thereby indirectly activating caspase-3, caspase-7 and regulating apoptotic cell death.
Zhang et al., 2019 [26]	Activates ataxia telangiectasia-mutated gene to upregulate p53 and downregulate p21 subsequently inhibiting CDK2 and cyclin E leading to G0–G1 phase cell cycle arrest. Also, induces apoptosis via caspase-3, cleaved caspase-9 and decreasing the levels of Bcl-2 and increasing Bax, leading to increased intracellular ROS.
Other cancer citations	
Huang et al., 2021 [22]	Autophagy and apoptosis were primarily responsible for cell death.
(Glioma), Young go et al., 2020 [25] (Squamous cell carcinoma)	

CBD and THC, but some data is available with other minor cannabinoids like CBG, THCV, CBN, and with terpenes. CBD specifically has been reported to inhibit cancer cell growth and proliferation in myeloma, leukemia, hepatocellular carcinoma, melanoma and breast cancer [13–16]. Δ^8-THC is a significantly stable and less potent isomer of Δ^9-THC and has been currently an area of extensive investigation. Unlike Δ^9-THC, it has lesser affinity for the CB1 receptors and therefore possesses no intoxicating psychoactive effects. However, both the compounds competitively (agonistic and antagonistic) bind to CB2 receptors with similar affinity. After binding to the CB1 receptors, both Δ^9-THC and Δ^8-THC activates the intracellular signaling pathway by activating the $G\alpha_{i/o}$ causing the inhibition of adenyl cyclase and recruitment of β-arrestin1 [17].

Among the other clinically relevant chemical constituents, CBD has gained interest for its efficacy in various neurological disorders. CBD has

Table 2
Studies conducted using drug loaded EVs for cancer treatment.

Cancer studied	Drug carrier	Outcome
Glioblastoma by Cui et al. [196]	Self assembled micelles (formed from tanshinone IIA and glycyrrhizic acid) loaded serum derived EVs	EVs showed extremely effective GBM cellular uptake and adequate intracellular drug release to induce apoptosis and dendritic cell maturation promotion and tumor-associated macrophage polarization induction
Breast cancer by Degirmenci et al., 2023 [197]	Lapatinib loaded healthy epithelial breast cells (MCF10 A) EVs	The drug loaded EVs showed increased potency and increased apoptotic rate in comparison to the free drug
Breast cancer by Zhao et al., 2022 [115]	DTX-laoded M1 macrophage-derived EVs	EVs induced naïve M0 macrophages which polarized to M1 phenotype causing M1 macrophage-dominant immunosuppressive microenvironment due to modulated mitochondrial function and showed improved efficacy of the loaded drug
Colorectal cancer by Hosseini et al., 2022 [198]	DOX-loaded anti-nucleolin aptamer (AS1411) surface-functionalized EVs	Functionalization of EVs showed increased uptake of the EVs in nucleolin-positive cells and showed increased therapeutic index of the drug and thereby suppressing the adverse effects
Breast Cancer by Pullan et al., 2022 [149]	DOX-loaded hypoxia-responsive lipid and neuropilin receptor agonist peptide (iRGD) modified bovine milk EVs	Modified EVs showed targeted release and penetration of the drug both in monolayer and spheroid cultures of four different types of triple negative breast cancer cells.
Anaplastic thyroid carcinoma by Wang et al., 2022 [199]	DOX and radioiodine-131 (131I) loaded iRGD HEK-293T EVs for chemotherapy/ irradiation combination therapy	Engineered EVs showed highly effective targeting and efficient drug delivery to the thyroid carcinoma cells and showed great potential of improving the overall efficiency of the chemotherapy and irradiation therapy
Osteosarcoma by Wei et al., 2022 [200]	DOX-loaded MSC derived EVs	EVs showed higher cytotoxicity against Osteosarcoma cells in-vitro and showed 6-fold decrease in tumor volume in MG63 cells xenograft nude mouse model.
Colorectal Cancer by Bagheri et al., 2020 [193]	DOX loaded MSC derived MUC-1 functionalized EVs	EVs showed efficient site delivery and increased anti-cancer activity of the drug in-vitro and suppressed tumor growth in BALB/c mice model of colon carcinoma as compared to the free drug.
Metastatic lung cancer by Kim et al., 2018 [192]	PTX-loaded macrophage derived EVs	Increased expression of sigma receptors was confirmed with EVs engineered with aminoethylanisamide-PEG.
Prostate cancer by Saari et al., 2015	PTX-loaded LNCaP and PC-3 cells derived EVs	Increased cytotoxic activity in the autologous cancer cells was observed.
Breast cancer by Tian et al., 2014	DOX-loaded immature dendritic cells derived EVs	EVs showed significant antitumor activity in both in-vitro and in-vivo models.
Breast cancer by Yu et al., 2019	Erastin-loaded human fetal lung fibroblasts derived EVs labeled with folate	EVs could increase the uptake of the low solubility drug (Erastin) into cancer cells and decreased proliferation and migration of the cells.
Human Breast Cancer by Schindler et al., 2019 [187]	DOX loaded HEK293 derived EVs	EVs showed increased uptake by cancer cells and increased intracellular accumulation of the drug along with its

(*continued on next page*)

Table 2 (*continued*)

Cancer studied	Drug carrier	Outcome
		increased potency over free drug and its liposomal formulation in-vitro.
Pancreatic cancer by Li et al., 2020	Gemcitabine-loaded pancreatic cancer cell derived EVs	Results showed considerable difference in the efficacy of the Gemcitabine EVs over other groups.
Human Ovarian carcinoma by Zhang et al., 2020	Cisplatin-loaded umbilical cord derived macrophage M1 and M2 EVs	EVs showed increased efficacy of the drug with more cytotoxic action at lower doses and lesser side effects of the drug.

also been used in clinics for its analgesic efficacy in cancer associated pain and is also used in palliative care for other diseases [18]. Additionally, it shows anticancer activity by inducing apoptosis and inhibiting cell migration in various cell types [19]. It has the ability to reduce the progression of various types of cancers including: melanoma, glioblastoma, breast, prostate, colon and lung cancers [20]. Unlike THC, it exhibits weak receptor affinity for cannabinoid receptors (CB1 and CB2) and may interact with other non-cannabinoid receptors of ECS: TRPV channels, PPARϒ and 5HT1A receptors. Affecting multiple molecular pathways, most of its anticancer activity is via regulation of reactive oxidative species (ROS) and endoplasmic reticulum stress [19]. While itself being a strong antioxidant, CBD has been reported to have strong cytotoxic action in glioblastoma by causing an early increase in ROS production and decreasing the levels of intracellular glutathione and thereby triggering oxidative stress [21]. Huang et al., 2021 in their study, to evaluate the underlying mechanism of the anticancer effects of CBD in glioma cells demonstrated the dose dependent inhibitory effect of CBD in different glioma cells lines: U118, MG, U251, U87MG, A172, and LN18. Their data from various in-vitro tests suggested that autophagy instead of apoptosis was primarily responsible for the cell death when treated with CBD; confirmed by the expression of autophagy biomarkers in the cells and increased number of autophagosomes [22]. In a study by Shrivastava et al., 2011, the cytotoxic effect of CBD was analyzed using various human breast cancer cell lines; MCF-7, MDA-MB-231, MCF-10A, MCF-7, SK-BR-3 and ZR75-1. They demonstrated CBD induced cell death in receptor independent and concentration dependent manner as the treatment with CBD decreased the viability of both estrogen positive and estrogen negative cells. They further analyzed that CBD induces cell death both with autophagy and apoptosis and this cytotoxic action is independent of the cannabinoid receptors and by increasing the cellular ER stress and inhibiting downstream AKT/mTOR/4EBP1 signaling [23]. Garcia Morales et al., 2020, explored the anti-inflammatory activity of CBD in reversing the IL-1β induced EMT in human breast cancer model using MCF-7 cells. The treatment with CBD blocked IL-1β induced signalling pathway (IL-1β/IL-1RI/β-catenin) and re-established the epithelial organization by increasing the expression of E-cadherin and β-catenin at the adherens junctions [24]. Go et al., 2020, analyzed the anticancer activity of CBD in malignant tumors of head and neck squamous cell carcinoma (HNSCC). They studied the cytotoxic and antitumor action of CBD alone in-vitro and in-vivo respectively, using four human HNSCC cells: FaDu, Hep2, SNU-899 and SCC15 cells against human oral keratinocytes (HOK) cells as normal control. Synergism was observed when using CBD in combination with standard anticancer drugs; cisplatin, 5-florouracil (5-FU) and paclitaxel (PTX) (FaDu cells). They also observed apoptosis and autophagy in cell death signaling in cells when treated with CBD. Their genome wide studies revealed the decreased expression of genes encoding MCM2, PARP1, and BRCA1 involved in cell proliferation, cell division and DNA repair in CBD-treated HNSCC cells [25]. Zhang et al., 2019, in their in-vitro evaluation of CBD using human gastric cancer SGC-7901 cells demonstrated the action of CBD against gastric cancer via G0–G1 phase cell cycle arrest and apoptosis with increased intracellular ROS production [26].

The most efficient cannabinoids, CBD and THC have also been analyzed in combinations with various cancer types [13,27–29]. Milian et al., 2013, in their study on analyzing the efficacy of THC and CBD in inhibiting cell proliferation and EMT in non-small cell lung cancer used THC and CBD separately and in combination (1:1 ratio). The IC_{50} value of the combination decreased to 12.94 from 27.25 to 37.31 μM for THC and CBD respectively for A459 cells. Similarly, IC_{50} changed from 30.6 to 39.78 μM for THC and CBD respectively to 8.04 μM in the combination with H460 cells and with H1792 cells it changed to 14.55 (combination) from 33.39 to 46.41 μM for THC and CBD respectively [13]. The combination was also found to significantly decrease the EGFR expression levels in A459 and H460 cells while sparing its effect in H1792 cells [13]. Andradas et al., 2021, in their study assessed the effect of combinations of THC and CBD in their in-vivo mouse models of medulloblastoma and ependymoma. They used three medulloblastoma cell lines D283, D425, and PER547 and two ependymoma cell lines IC-1425EPN and DKFZ-EP1NS and observed CBD and THC to have dose inhibiting 50% of cells (ED_{50}) to be in low micromolar range. They further combined THC and CBD in the treatment to analyze if both the agents synergize to induce their cytotoxic effect and found significant synergism of the compounds only in D283 cells [28].

4. Molecular mechanisms of CBD

Significant work has been done to understand the mechanisms of CBD and various pathways have been proposed and summarized in Fig. 1. The efficacy of CBD has also been analyzed for its action against cell invasion and metastasis. Ramer et al., 2012, demonstrated for the first time the role of intercellular adhesion molecule-1 (ICAM-1) in increasing the tissue inhibition of metal-proteinases-1 thereby inhibiting the cancer cell invasion, where ICAM is a transmembrane glycoprotein, (also known as CD54), has a crucial role in trafficking of inflammatory cells and has traditionally been known to do so by acting as the adhesion molecule. However, new evidence presents a controversial status of whether the upregulation or downregulation is involved in metastasis. Ramer et al., 2012, principally focused on the antimetastatic action of CBD in-vivo and anti-invasive action in-vitro and their results revealed for the first time that increased ICAM-1 levels with CBD treatment resulted in decrease in metastasis and tumor cell invasion observed with lung tumor xenografts, lung tumor cells, and primary tumor cells (isolated from the brain metastasis of a patient who had NSCLC) [30]. The treatment of CBD has also been correlated with its ability to downregulate the gene expression of ID-1, an inhibitor of basic helix-loop-helix transcription factor, potentially known for their role in regulating the metastatic potential of various cancers. The overexpression of ID-1 in breast cancer cells has been found to be significantly related to the metastasis of human breast cancer cells. A study evaluated the effect of CBD to reduce the expression of ID-1 in MDA-MB-231 cells which was confirmed with RT-PCR. At the same time, the reduction in the breast cancer cell invasiveness was also studied. The authors also reproduced the results in another metastatic breast cancer cell line, MDA-MB436 [31].

Kalvala et al., 2022, used THCV and CBD alone and in combination with Doxorubicin (DOX) against resistant MDA-MB-231 xenografts in athymic nude mice. They demonstrated that the treatment with CBD and THCV significantly increased the cytotoxicity of DOX against resistant MDA-MD-231 cells in 3D tumor cultures and also in-vivo. Their transcriptomic and proteomic analysis showed alteration in the expressions of NLRP3, TGFβ, P38-MAPK, PDL-1 and AMPK induced apoptosis involved in improving DOX chemosensitivity against resistant MDA-MB-231 tumor. Further, CBD and THCV were shown to counteract histone modifications and their subsequent effects on DOX, resulting in chemosensitization against MDA-MB-231 resistant cancers [39].

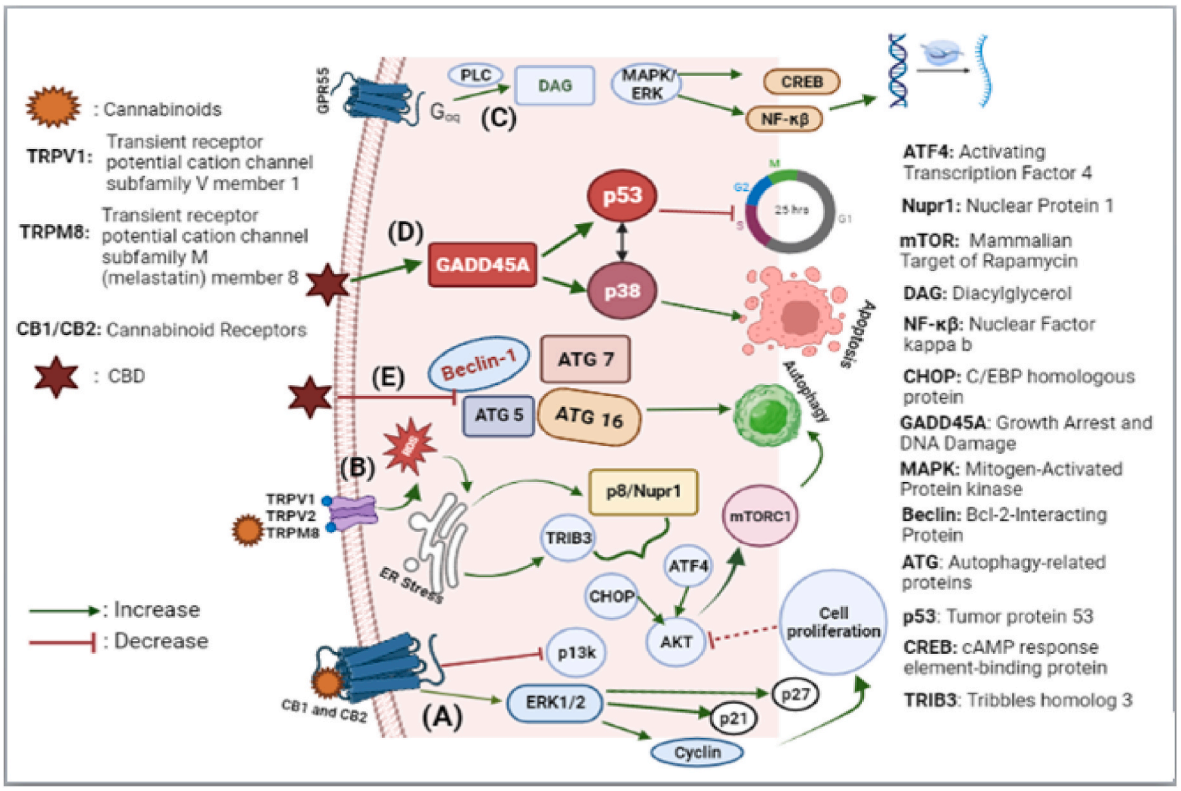

Fig. 1. Representation of downstream molecular mechanisms and signaling pathways of cannabinoids when they interact with their receptors (cannabinoid and non-cannabinoid receptors). Interaction of cannabinoids with CB1 and CB2 receptors inhibits the P13k pathway leading to decrease in Akt and ultimately inhibiting cell proliferation. It also activates ERK1/2 leading to the stimulation of p27 and p21 followed by increase in pRb leading to decrease in cyclins (D&E) and cyclin kinases ultimately leading to cell cycle arrest. Activation of non-cannabinoid receptors: TRPV1 and TRPV2 causes the formation of reactive oxygen species (ROS) which causes the increase in ER stress with increase in the levels of stress proteins p8/Nupr1 and TRIB 3 which activates upregulation of ATF4 and CHOP proteins and decrease in Akt. Decrease in Akt further downregulates the mTORC1 signaling causing autophagy. Stimulation of GPR55 (non-cannabinoid) receptors leads to the activation of PLC-DAG pathway which further causes the activation of MAPK/ERK signaling and this results in the gene transcription by activation of transcription factors CREB and NF-κß.

5. A deep dive in exosome biogenesis

A sophisticated network of enzymatic reactions and signal transductions is involved in the synthesis of exosomes as shown in Fig. 2. After internal endosome budding, early endosomes develop into multivesicular bodies (MVBs) or late endosomes, creating intra-luminal vesicles (ILVs) that shed into exosomes [40]. Typical biogenesis of exosomes involves the following processes: An early secretory endosome is formed by invagination of the cytoplasmic membrane followed by the payload sprouting inward to form ILVs. Then, the late endosomes mature by acidification eventually leading to extracellular release of ILVs as exosomes (EVs) by fusion with the plasma membrane which is governed by endosomal sorting complexes required for transport (ESCRT complex). The AAA ATPase Vps4 complex with the four distinct

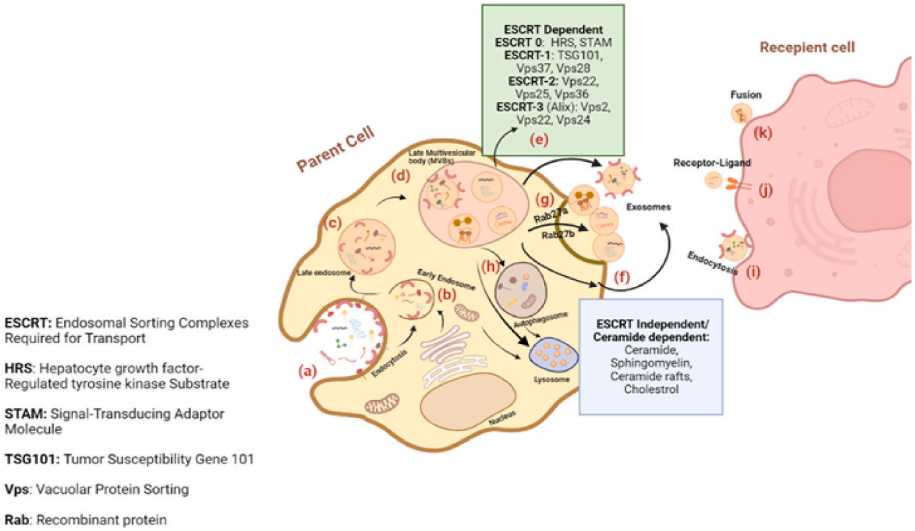

Fig. 2. Schematic representation of exosome biogenesis and release from the cells. (a) The exosome formation initiates with endocytosis, starting with the inward budding of plasma membrane along with inward movement of the extracellular contents. (b) It further involves the cargo sorting (invagination of cytosolic proteins and other components to form early endosomes (c) followed by formation of late endosomes and (d) Multivesicular bodies (MVBs), where MVBs consists of intra-luminal vesicles (ILVs) formed as a result of the invagination of late endosomal membranes via different mechanisms; (e) ESCRT dependent (f) ESCRT independent. These ILVs are released from the parent cells into the extracellular spaces using (g) Rab proteins. (h) The ILVs also undergo degradation forming autophagosome followed by lysosome fusion. After getting released extracellularly, the exosomes interact with recipient cell using different mechanisms (i) endocytosis (j) exosome receptor-ligand interaction and (k) direct fusion with the cell.

protein complexes ESCRT-0, –I, –II, and –III make up the ESCRT. Exosome biogenesis has frequently been referred to as either an ESCRT-dependent or ESCRT-independent/Ceramide dependent mechanism. It is unclear how this ESCRT-independent mechanism works, but it might be brought on by the ceramide microdomains that form and then grow into bigger domains that encourage membrane budding [41–43]. EVs have the ability to merge with recipient cell plasma membranes after being released from the cell surface, releasing their contents into the cytoplasm. As an alternative, EV surface proteins may interact with destination cell surface receptors to trigger intracellular signaling. Therefore, a key determining factor in the impact of EVs is their cargo [44,45]. EVs include cell-specific proteins and lipid components that are a reflection of their biological source origin depending on the cell from which they were produced [46].

EVs are abundant in cytoskeleton components, signal transducers, tetraspanins, lipids, RNAs, and DNAs, as well as enzymes, transcription factors, major histocompatibility complex (MHC), and heat shock proteins (HSPs) [47,48]. EVs vary in size and biomolecular inclusions, although all kinds share some basic elements. Cholesterol, sphingomyelin, glycosphingolipids, phosphatidylcholine, phosphatidylserines, phosphatidylethanolamines, and saturated fatty acids are some of the substances that make up lipid components of EVs [49,50]. Particularly, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), transfer RNAs (tRNAs), ribosomal RNA (rRNA) fragments (such 28S and 18S rRNA subunits), and messenger RNAs are among the several types of RNAs (mRNAs) in EVs [51]. Retrotransposons, single-stranded DNA, double-stranded DNA, and mitochondrial DNA (mtDNA) are additional exosomal cargo components. MHC class I and II molecules, cytokines, ESCRT complex, tetraspanins, glycosylphosphatidylinositol-anchored proteins, rabs, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNARES), and flotillin make up the protein components [52]. To preserve composition parity with the cell membrane and aid in the sequestration of soluble ligands, the plasma membrane proteins form the vesicle membrane. Exosomal proteins are essential for the following processes: a) antigen presentation; b) cell adhesion; c) cell structure and motility; d) control of stress; e) transcription and protein synthesis; and f) trafficking and membrane fusion [53,54].

EVs are secreted by almost all types of cells in the body. EV release in extracellular media has been reported from a variety of cell types in-vitro as well as in biological fluids described earlier by other researchers [55]. It has been proposed that all EVs, whether released by cells in culture into their media or by distinct organs into related body fluids, engage in intercellular communication, supporting or impairing (in the case of malignancy) numerous physiological processes [56]. EVs are essential for the transportation of bioactive molecules, immunological response, antigen presentation, protein quality regulation, protein translation, mRNA regulation cellular homeostasis, and remodeling of the extracellular matrix [57–60]. EVs are also implicated in a number of pathogeneses, such as the development of autoimmune diseases, tumor metastasis, and the spread of infections [61]. EVs play a variety of biological functions, including immunological regulation, which controls the production of antigens and the immune system's response to them [62–65].

6. Isolation, drug loading and characterization of exosomes

6.1. Isolation

EVs can be isolated from bodily fluids or cell culture supernatants using established procedures such as ultracentrifugation, ultrafiltration, immunoaffinity capture, precipitation, chromatography, and microfluidics-based techniques [66]. One of the most widely used and documented methods for isolating EVs is ultracentrifugation which is regarded as the industry standard. According to estimates, more than half of all exosome separation methods use ultracentrifugation [67]. While ultracentrifugation continues to be the "gold standard" for EV extraction, combining it with additional isolation techniques can result in a greater yield. EVs yield by ultrafiltration is higher than those from ultracentrifugation [68]. Sequential centrifugal ultrafiltration (SCUF), a highly sophisticated ultrafiltration technique and has been utilized to separate microvesicles from a human colon cancer cell line to extract very pure EVs [69]. Sometimes, to concentrate EVs from enormous quantities of original material into small volumes that can be employed in further purification processes, ultrafiltration is frequently used as the initial step [70]. Ultrafiltration gives better recovery of EVs which are less than 100 nm in diameter and is time saving and economical [68]. Given that unique surface proteins may be found to distinguish EVs from other particles, immunoaffinity capture is a valuable method in the extraction of EVs. It generally functions by using antibodies that specifically latch to certain proteins on the surface of EVs to create a form of filter that can later be eluted for additional use [71]. EVs have several membrane proteins on their surfaces, including CD9, CD63, ALIX, and Ep-CAM, which can be enhanced using magnetic beads with antibody coatings. Compared to ultracentrifugation or ultrafiltration, immunoaffinity capture has a better efficiency of isolation [72]. Also, microfluidics devices are the best method for isolating exosomes from other nanometer-sized particles because they enable quick, accurate, and cost-effective isolation procedures [73]. Currently, size-based separation, immunoaffinity and dynamic separation are fully integrated with commonly used microfluidics technologies. The ExoTIC gadget, a new EV isolation technology has unquestionable benefits, such as its high yield, purity, and effectiveness which has rapidly expanded its popularity. The ExoTIC device is more suitable for extracting EVs from serum or other physiological fluids than ultracentrifugation [74,75]. Rewards of microfluidic-based EV isolation comprise specificity, reproducibility, short isolation times, and lower isolation costs [76]. Another popular method uses the mechanism of precipitation and chromatography which contrasts with the aforementioned isolation methods, which primarily relies on the application of polymers such as PEG and size exclusion respectively to extract EVs. Precipitation, for example, is used in System Biosciences' ExoQuick which is a proprietary polymer that can be used to isolate EVs for a variety of downstream uses. Izon's qEV columns offer EV recovery and purity at low levels and use size exclusion chromatography to efficiently isolate highly purified EVs by separating EVs from soluble protein [77–79]. There are various drawbacks that restrict some exosome isolation and purification techniques from fully satisfying all requirements. The separation effect of a combination of multiple isolation techniques may be superior to that of a single technique. These methods in combination with the methods mentioned above are ideal for EV isolation.

6.2. Loading

EVs have the potential to be effective natural delivery systems for nucleic acid and small molecule therapeutics. Different techniques, such as sonication, electroporation, transfection, incubation, extrusion, saponin-assisted loading, freeze-thaw cycles, thermal shock, pH gradient approach, and hypotonic dialysis, have been used to load exogenous chemicals into EVs. Numerous studies have demonstrated the efficacy of sonication approach; in some instances, it has resulted in multiple layers of drug encapsulation, some of which incorporate inside the EVs and some within the membrane, resulting in multiple drug release, where the membrane-bound portion is released more quickly, and the institutionalized drug is released over a longer period of time [80,81]. A group of researchers compared a modified transfection technique to traditional electroporation, and they observed that the amount of miRNA introduced into exosomes in both cases was comparable [65]. EVs can be loaded with cargo using commercially available transfection reagents [82–84]. A study in the last decade used this technique to treat 9 L gliosarcoma cells by transfecting bone marrow stromal cells with miR-146b and then harvesting the EVs. The collected EVs that were

produced after the transfection revealed elevated miR-146b expression and were demonstrated to inhibit the formation of glioma in rat models [85]. It is important to point out that there is, nonetheless, no certainty on which method offers more benefits for encapsulation of drugs yet. This review has covered the procedures currently in use for both conventional and modified EV loading strategies. Here, we concentrated on the benefits and drawbacks of each approach while talking about specific findings from significant reports. Any method that is used in practice should be evaluated thoroughly to determine which is the most effective, considering things like drug characteristics, effectiveness, and effects on subsequent experiments to be followed.

6.3. Characterization

Due to the tiny size of EVs and simplicity of sample preparation, transmission electron microscopy (TEM) is recognized as the gold standard for studying the morphology of EVs by electron microscopic imaging [86]. An advanced version of TEM is Cryo-EM which involves sample preparation at cryogenic temperature that allows a wide range of extracellular vesicles with different sizes and morphologies, as well as lipid bilayers and vesicular interior structures, to be seen [87,88]. Scanning electron microscopy (SEM) is an alternate method that has just lately become popular [89]. According to a study, despite the morphological variations seen with the two EMs, these methods determine EV sizes similarly. SEM offered a quicker substitute for TEM for imaging EVs native morphology and determining sample purity [86]. EV topography can be examined with atomic force microscopy (AFM) at a high resolution of less than 1 nm. In a study, antiCD63 IgG functionalized AFM tips were used in conjunction with force spectroscopy to identify cell-type-specific markers, such as CD63, on individual EVs [90]. CBD was loaded in human umbilical cord mesenchymal stem cell (hUCMSC) derived EVs and characterized by AFM and it was observed that there was an increase in height peak and surface roughness significantly with CBD loaded EVs vs plain EVs [91]. The method designated as nanoparticle tracking analysis (NTA) can size and count nanoparticles, including those secreted by cultured cells [92]. This method for evaluating the average quantity and size of EVs produces reliable and accurate results, which are supported by additional techniques like SEM [93].

Using an optimal configuration of a commercially available high-end flow cytometer, it enables high-resolution quantitative and qualitative examination of individual cell-derived EVs [94]. According to several investigations, EVs with sizes between 300 and 500 nm can be detected semi-quantitatively using their surface markers [95]. However, the limited resolution of flow cytometry makes it an unreliable method for EV detection. Parallel to flow cytometry, ImageStream enables statistical analysis of many cells based on their fluorescence characteristics. Additionally, using obtained cellular images that integrate numerous morphometric and photometric characteristics of the analyzed cells. ImageStream enables extensive morphometric cellular analysis [96]. The instrument's novel characteristics provide additional analytical capabilities and opens a wide range of potential applications outside the realm of flow cytometry [97]. Other techniques like BCA protein assay and western blotting are employed for protein quantification and analysis for specific EV markers. Since characterization is an important parameter for EVs, it must be pointed out that any approach that is put into use should be thoroughly assessed to see which is the most productive, taking into account factors like drug properties, drug loading, productivity, damage to the EV membranes etc.

7. Anticancer potential of EVs from different sources

It is generally accepted that EVs display a wide range of functions depending on their source. Almost all cell types, including lymphocytes, dendritic cells, epithelial and endothelial cells, mast cells, and neurons, release EVs. Additionally, they are present in most bodily fluids, such as blood, saliva, urine, breast milk, amniotic fluids, ascitic fluid, and the culture medium for practically all cell types [98,99]. Approximately 85% of the EVs that circulate come from immune cells like dendritic cells, lymphocytes, and platelets. EVs can be produced and released by a variety of cells, including epithelial cells, macrophages, mast cells, reticulocytes, neurons, B-cells, T-cells, oligodendrocytes, Schwann cells, and tumor cells as shown in Fig. 3 [100,101]. Different EVs from varied cell types have different tumor-regulating characteristics [102]. EV vesicular makeup, which is an indicator for pathophysiological roles in any organism, is totally reliant on the parental cells and fluids [103]. Immune cells are essential for controlling the growth, invasion, and metastasis of tumors as well as for inducing immune reactions at tumor environments [104]. Furthermore, immune cells such as macrophages, dendritic cells (DC), T cells, B cells, and natural killer (NK) cells can also constitutively release EVs that can alter their functions [105,106]. EVs released from immunocytes play a crucial role in altering the tumor microenvironment and enhancing therapeutic efficacy in anticancer treatment because of the immunological properties of the originating cells [107]. The anticancer mechanism of EVs generated by various immune cells, including DCs, macrophages, T cells, B cells, and NK cells, has been briefly discussed here.

7.1. DC-derived EVs

DC-derived EVs contain functional MHC-peptide complexes along with a variety of other immune-stimulating components that together promote immune cell-dependent tumor rejection and have attracted a lot of attention as immunomodulatory antitumor agents [108,109]. Furthermore, tumor-infiltrating DCs can activate memory T cells to drive differentiation, deliver tumor-specific antigens to effector T cells, boost the therapeutic potential of inhibitors of immune checkpoints and ultimately prevent tumor recurrence [110]. EVs from DCs induced NK cell proliferation and activation under the control of IL-15R and NKG2D, respectively, with subsequent anti-metastatic effects carried out by NK1.1+ cells. Human DC-derived EVs have functional IL-15R, allowing NK cells to proliferate. Human DC-derived EVs, as opposed to immature DC, have NKG2D ligands on their surface, resulting in a direct interaction of NKG2D and NK cell activation ex-vivo [111]. Also, EVs released by DCs were more effective at stimulating the generation of antigen-specific IgG and activating $CD8^+$ T cells [112]. The ability of DC-derived EVs to directly destroy tumor cells and activate NK cells via tumor necrosis factor (TNF) superfamily ligands has also been reported [113]. In another study by Tian et al., 2014, the authors explored the effect of using immature dendritic cells (imDCs) derived EVs to deliver DOX to MDA-MB231 tumor tissues in BALB/c nude mice. They specifically used the imDCs EVs because they are devoid of various immunogenic markers namely CD40, CD86, MHC-I and MHC-II. Further these EVs were subsequently engineered with a well-characterized exosomal membrane protein, lysosome associated membrane glycoprotein 2b (Lamp2b) and also fused the EVs with iRGD targeting peptide for αv integrin targeting. The in-vitro and in-vivo anticancer efficacy of the loaded EVs showed a significant antitumor activity as compared to the unloaded EVs, plain DOX and control groups) [114]. In a previous study, it was seen that although both immature and mature DCs released EVs, mature DCs (mDCs) derived EVs were able to stimulate immune responses more than imDC EVs due to their higher expression of MHC-I and II, CD80, CD86, and ICAM [115]. EVs generated from DCs treated with interleukins and interferons have been reported to decrease inflammatory autoimmune responses and had improved immunostimulatory properties [111,116]. Alpha-fetoprotein (AFP)-expressing DCs were used to produce novel EVs, which showed that the tumor microenvironment was noticeably improved in HCC mice treated with these EVs as evidenced by significantly stronger $CD8^+$ T lymphocyte activation and diminished immunosuppressive microenvironment [117].

7.1.1. Macrophage-derived EVs

M1-polarized macrophages produce pro-inflammatory and

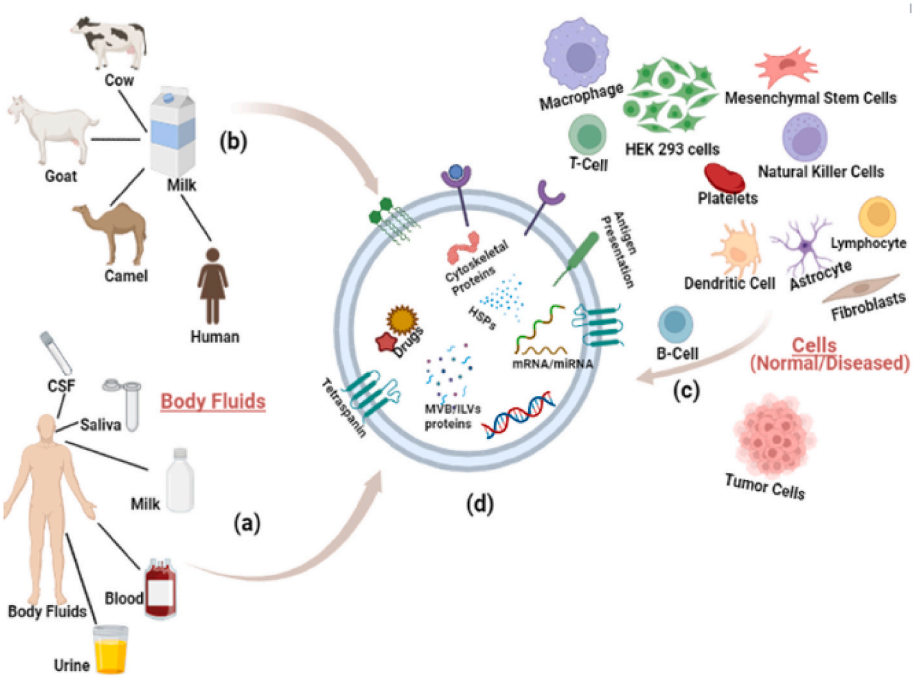

Fig. 3. Sources and composition of exosomes: (a) Human body fluids as a source of exosomes (saliva, CSF, blood, urine, milk). (b) Goat, camel, cow, human breast milk are most common and most studied sources of milk exosomes. (c) Various cells both normal (dendritic cells, macrophages, NK cells, mesenchymal stem cells, platelets, astrocytes, lymphocytes, fibroblasts, HEK 293 cells, T-cells, B-cells) and diseased cells (tumor cells) are used to isolate exosomes. (d) Representation and composition of a typical exosome.

immunostimulatory cytokines, such as interleukin-12 (IL-12), interferon-γ (IFN-γ), and TNF-α [115]. EVs released from M1-macrophages, can be used as immunological boosters for cancer vaccines. According to a study, these EVs improved the efficacy of the Trp2 vaccine when it was loaded with lipid calcium phosphate (LCP) nanoparticles (NPs), as well as the response of cytotoxic T cells to the antigen. In a melanoma growth inhibition trial, the EVs were found to be more effective immune enhancers when combined with the LCP NPs vaccination than CpG oligonucleotides [116]. In another study, macrophage-derived EVs increased the antitumor effectiveness by promoting inflammation through the caspase-3 signaling pathway. Additionally, in-vivo tumor-bearing mice were efficiently protected from tumor progression by these EVs. In another study, paclitaxel (PTX) was loaded onto macrophages, particularly M1 macrophages, which demonstrated to overcome drug resistance mechanisms and have potent antitumor effects. PTX-loaded EV therapy had greater antitumor efficacy than either paclitaxel or exosome alone treatment [117]. In another study, umbilical cord derived macrophages M1 and M2 EVs were used to deliver cisplatin in human ovarian carcinoma cells. It was demonstrated that both M1 and M2 macrophages derived EVs showed increased cancer cell toxicity of the drug, while delivery of cisplatin with M1 derived EVs decreased its cisplatin IC_{50} more significantly with A2780 cells. The results signified that umbilical cord macrophage derived EVs encapsulated cisplatin is more cytotoxic at low concentrations. This effect can lead to the decrease in the drug toxicity and increased efficacy [118]. EVs derived from infected macrophages may present as antigens and activate T cells that are specific for that antigen [119]. The ability of macrophage EVs to partially transfer macrophage antigens to DCs in a ceramide-dependent manner for powerful antigen presentation and improved T-cell responses has also been demonstrated [120]. M1 macrophage EVs were typically used in combination with other anti-tumor immunotherapy techniques. For instance, Choo et al. created M1 macrophage-derived nanovesicles to enhance anti-PD-L1 antibody (aPD-L1)'s anticancer activity [121]. All of the aforementioned studies showed that macrophage derived EVs had potent anti-tumor immunotherapy characteristics and could function as adjuvants for impacting the TME.

7.2. T cell derived EVs

The $CD4^+$ and $CD8^+$ T cells are crucial for developing an effective immune response in the tumor microenvironment [122]. EVs produced by T cells have been shown to activate T cells and boost their antitumor effectiveness by inducing the production of granzyme B and IFN- γ [123]. Target cells are killed by lethal components (like granzymes and perforin) present in EVs as a result of the functional interaction between the antigen-specific receptors (TCR) and the responsive antigen/MHC complex [124]. CARs, CD3, CD8, and TCRs were among the surface membrane molecules of the parental T cells that were preserved by CAR-T cell-derived EVs in one investigation. These biological characteristics allowed the CAR-T cell-derived EVs to efficiently target triple-negative breast cancer cells both in-vitro and in-vivo. As a result, EVs from CAR-T cells have high anticancer potential against solid tumors [125].

7.3. B cell derived EVs

EVs produced by B cells are involved in both T-cell activation and antigen presentation [4]. It has been reported that EVs released from B cells carry histocompatibility MHC complexes, which can stimulate $CD4^+$ and $CD8^+$ T cells to multiply and secrete cytokines, hence promoting the cytotoxic T lymphocyte response [126]. EVs released by B cells can increase T cell development and T_{H2}-like cytokine production, which may help T cell-based immune response initiation in cancer therapy [127].

7.4. NK cell derived EVs

Several cell death mechanisms, including endoplasmic reticulum stress-induced apoptosis and caspase-dependent/-independent apoptosis, can be triggered in cancer cells by NK cell derived EVs [128]. EVs from NK cells have been used to manage antitumor immunity in cases of aggressive melanoma, gastric cancer, and colon cancer [129, 130]. Researchers used NK-derived EVs as an anticancer therapy on cancer neuroblastoma (NB) cells to examine the efficiency of these EVs on cancer cells. Both in-vitro and in-vivo, NK-derived EVs showed cytotoxicity against NB cells, but naive NB-derived EVs acted as tumor

promoters by creating a microenvironment that supports tumor growth [131]. Hsp70 is highly expressed in EVs generated from hepatocellular carcinoma (HCC), which also improve tumor immunogenicity and boost the efficiency of natural killer cells by upregulating the suppressive receptor CD94 and downregulating the stimulating receptor CD69 [65].

7.5. Tumor cell derived EVs

EVs released in the tumor microenvironment (TME) can either stimulate immune cells to produce antitumor effects or can counteract those effects [132,133]. Tumor derived EVs (TDEs) and their parent cancer cells' cargo are remarkably similar [134]. EVs play a variety of roles in the development and spread of tumors, including those involving tumor angiogenesis, tumor cell proliferation, migration, metastasis, and apoptosis [135–138]. Tumor cells in the TME release EVs that may promote tumor growth when nutrients are deprived [139,140]. In a study by Li et al., 2020 pancreatic cell derived EVs were used for the autologous delivery of the drug to the cancer cells. EVs from Panc-1 cells and from A549 cancer cell lines were isolated to compare the autologous uptake of EVs which was found to be significantly higher in case of autologous cells. In their xenograft tumor model (BALB/c nude mice) and in-vitro model of pancreatic cancer, they showed significant difference in the efficacy over other groups [141]. Further, Saari et al., 2015 used EVs derived from prostate cancer LnCaP and Pc-3 cells. Their study was oriented towards the ability of EVs derived from diseased cells to show their therapeutic activity against the same cancer. They labelled their EVs and loaded PTX with DiD lipophilic dye and OregonGreen dye respectively to navigate the uptake of the EVs by the autologous cancer cells and subsequently demonstrated the same using flow-cytometry based assays. With the fluorescent labeling of EVs and drug, they were also able to demonstrate the endocytic pathway to explain the cellular release of PTX. Noteworthy, the PCa EVs with no drug cargo increased the cell viability when incubated with their parent cell lines, while the PTX loaded EVs showed significantly increased cytotoxic action of PTX [142].

7.6. Milk derived EVs

Milk EVs were described for the first time in 2007 and gained enormous attention because of their rather economical availability and promising therapeutic approach to deliver anticancer agents. To date, milk EVs have been isolated from human, camel, rat, horse, sheep, panda, porcine, bovine, yak and goat milk. Unlike the tendency of protein component of cancer cell and EVs derived from other sources to trigger immune responses when administered systemically, milk derived EVs bypasses the issues of immune response with added advantage of obtaining EVs in scalable quantities [133,134]. Lipids and proteins comprise the principal structural components of milk EVs owing to their physiological action where the protein content may vary based on age and nutrition intake of the host, stages of lactation, physical activity and any disease condition (if present) [135]. Free non-loaded EVs derived from camel milk showed increased apoptotic markers (increased caspase-3 activity, upregulated Bax expression and downregulated Bcl-2 expression) as well as decreased inflammatory and oxidative stress (increased levels of MDA and decreased levels of catalase, superoxide dismutase (SOD), glutathione peroxidase) overall contributing to the significant decrease in the progression of breast tumors [136]. Munagala et al., 2016, for the first time reported the use of bovine milk derived EVs as potential drug delivery vehicles for both, hydrophilic and lipophilic agents. They demonstrated the absorption and biocompatibility of milk EVs in in-vitro and in-vivo model of human cancers. They particularly used A549 human lung cancer models to support their hypothesis. The same research group also obtained a patent (US10420723B2) and put forth the idea of loading these EVs with macromolecules including siRNAs, miRNAs, cDNA, plasmid DNAs of interest, antibodies, peptides and various other tumor targeting ligands [143,144]. The inventors further used milk colostrum as a source of EVs owing to their higher levels of immune-related miRNAs correlating to their higher immune-boosting effects than the mature milk and eventually obtained another patent (US10166259B1) in which they showed that their EVs could encapsulate various lipophilic and hydrophilic drugs/agents as payloads [145,146]. Further, it is expected that milk EVs can be further modified by engineering with various ligands for superior targeting [147–150]. The drug loaded milk EVs were analyzed for the first time in in-vitro and in-vivo models of human lung cancer. The drug loaded EVs showed increased efficacy as compared to the free drug in both cell culture and xenograft model of lung tumors [134]. The biocompatibility and uptake of the milk EVs by the cancer cells has also been demonstrated along with versatility of the drug molecules they can carry as payload [117,134,137,138]. Standard anticancer therapy like PTX, docetaxel and natural bioactives with anticancer properties like curcumin and anthocyanidins were loaded in raw bovine milk derived EVs and demonstrated to have increased antiproliferative activity in various human cancer types [134,139,140]. Similarly, the delivery of celastrol, a plant-based triterpenoid, using milk EVs showed increased antitumor activity than its free form thereby circumventing its restricted use due to poor bioavailability and high toxicity. Interestingly, EVs derived from camel milk have their own anticancer properties with overall increased efficacy and decreased immune response as compared to the inherent anticancer activity of camel milk against human breast cancer cells (MCF-7 cells) [136]. However, even after the use of milk EVs as nanocarriers for anticancer agents by various investigators, the use of milk EVs is still in its early stages and the issues related to the lack of specificity for targeted delivery needs to be addressed and more research work is needed to be done in this area [141].

8. Contribution of EVs' cargo in anticancer activity

The first "proteomic" studies of the protein content of EVs secreted by dendritic cells were conducted in the late 1990s, and these were quickly followed by many similar studies using EVs purified from various sources. In addition to western blotting, these studies used mass spectrometry to identify proteins in a complex mixture [151]. One of the main proteins discovered by proteomics on EVs released by dendritic cells is milk fat globule-EGF-factor VIII (MFGE8), also known as lactadherin and when it binds to cancer cells, encourages macrophages that express v3 and v5 integrins to induce phagocytosis [152,153]. Almost all cell types secrete EVs, which contain MHC class I molecules that may cause CD8 T cell activation [154]. The situation with EVs secreted by antigen presenting cells (APCs) is different. EVs from DCs have been shown to include functional MHC I-peptide complexes by a few studies to be able to activate CD8 cytotoxic T lymphocyte (CTL) clones on their own [155,156]. The production of IFN-γ and granzyme B, as well as the activation and antitumor efficacy of T cells, have all been observed to be stimulated by EVs generated from T cells [157].

In addition, careful analysis of immune responses in the first cancer patients who received EVs from their own DCs revealed that these EVs had a boosting influence on the patient's NK cell activity [111]. These EVs were shown to include complexes of IL-15 and its receptor, IL-15R, as well as NKG2D ligands. This allowed for simultaneous activation of NKG2D and IL-15 transpresentation to NK cells, activating their cytotoxic potential. NK cell derived EVs possess anticancer activity by two pathways. The first involves the membrane-disrupting protein perforin and a family of structurally related serine proteases working along with cytoplasmic granule toxins (granzymes). The second pathway uses target-cell death receptors, such as Fas, through their specific ligand, FasL, which triggers caspase-dependent apoptosis [158,159].

One of the main immunological checkpoints, Programmed Cell Death 1 (PD-1), is expressed on a variety of immune cells. In particular, the interaction between PD-1 on the surface of T cells and PD-L1 on the surface of tumor cells can prevent T cells from killing cancer cells. PD-1-expressing EVs released by activated T cells have been found to compete

with PD-L1 on the surface of tumor cells, reducing the binding of PD-1 to PD-L1 on the surface of T cells, and decreasing T cell dysfunction [160]. Additionally, miR-16–5p can be carried by EVs and released by macrophages to decrease the expression of PD-L1 in gastric cancer cells. This will lessen the inhibitory effect of immunological checkpoints on T-cells and increase their capacity to activate and destroy cancer cells [161]. In another study, gastric cancer (GC) cells took up EVs derived from GC fibroblasts, which had anticancer effects on GC. Exosomal miRNA-34 and miRNA-139 was responsible for reduced tumor growth in-vivo and decreased the proliferation and invasion of GC cells in-vitro [162,163]. EVs derived from natural killer (NK) cells containing the tumor suppressor miRNA-186 are cytotoxic to MYCN-amplified neuroblastoma cell lines. These EVs ability to cause cell death was partially reliant on miR-186 expression [159]. EVs harboring miR-21 sponge constructs have the potential to upregulate the target genes PDCD4 and RECK of miR-21 and inhibit their malignant behavior by downregulating the expression of miR-21 in the glioma cell lines U87-MG and C6 [164].

EVs produced by mast cells contained immune-related molecules such as MHC II, CD86, LFA-1, and ICAM-1 that prompted the proliferation and cytokine production of B and T lymphocytes. Additionally, EVs containing endogenous heat shock proteins and exogenous antigens can cause specific IgG1 and IgG2a antibody responses in mice. Further, EVs can effectively promote DC cell maturation, perform the role of an antigen presenter by presenting a particular antigen to T cells, and activate T cells [165,166].

9. EV delivery of anticancer drugs

Numerous attempts have been made over the past few decades to overcome the challenges of using conventional anticancer drugs by the development of nano-drug delivery systems with improved efficacies and decreased toxicities [167–173]. These nano-drug delivery systems namely liposomes, nanoparticles etc. possess the targeted delivery and selective accumulation in tumor cells due to their enhanced permeability, active transport intake, high retention effect evading their toxic effects towards noncancerous cells [160,161,163–174]. The size, surface characteristics and increased permeability and retention of these nanocarriers also increase the half-life of anticancer drugs or nucleic acids targeted for their cytotoxic activity. The targeted delivery with nanocarriers allows the drug to target the cancer cells exclusively while sparing the noncancerous cells. Additionally, nanocarriers also help in encapsulating the poorly soluble drugs and their systemic delivery. Unfortunately, despite the aforesaid advantages, these nanocarriers have various inadequacies like a) inability to overcome the reticuloendothelial system b) inability to cross the blood brain barrier and c) toxicity caused by the excipients used to make the formulation. Clinical use of the most efficacious anticancer drugs like DOX is limited due to their poor biocompatibility and serious adverse effects of bone marrow suppression and cardiotoxicity. Similarly, PTX represents low bioavailability and dose-dependent toxicity. The efforts made using nanoparticle based methods like Abraxane to enhance biocompatibility and efficacy of these anticancer agents were also less effective due to their formulation associated side effects like oxidative stress [186].

With the aforementioned disadvantages of the conventional nano-drug carriers, the naturally derived drug delivery vehicles like EVs have gained considerable attention due to their ability to overcome these challenges of synthetic nanocarriers. Regardless of their role in cancer metastasis and progression, EVs can be used as potential drug delivery systems with added advantages and have ability to possess superiority over liposomal formulations in terms of increasing drug's potency and efficacy [175–177,187]. EVs being secreted by almost all the cells of the body and due to their role in mediating inter and intra-cellular signaling also have several advantages: a) ability to deliver both hydrophobic and hydrophilic drugs in the body b) ability to cross the blood brain barrier c) least or no immunogenicity and d) due to their small size and cellular origin, they are flexible to incorporate and deliver macromolecules like miRNA, DNA, siRNA. Further EVs have been engineered to incorporate different therapeutic agents including miRNAs, chemotherapeutic drugs and proteins [178–181,188,189]. Various studies have been reported to have successfully load and deliver the chemotherapeutic agents namely PTX, DOX, Docetaxel, 5-fluorouracil, cisplatin, curcumin etc. which have been further investigated in various in-vivo and in-vitro cancer models with potentially higher ability to target the cancer cells, as compared to nanodelivery systems [59,181–184,190]. The EVs derived from human platelets cells activated with ADP have also been used to deliver DOX to breast cancer cells and tumors due to the tendency of platelets to bind and form agglomerates with the cancer cells. The MDA-MB-231 cells showed decreased cell viability, colonogenic ability and migration when treated with DOX loaded human platelet derived EVs [185,191]. In another in-vitro model of human breast cancer cells (SK-BR-3 and BT20 cells), the HEK293 cells derived EVs loaded with DOX showed improved uptake of the exosomal DOX with increased intracellular accumulation and potency as compared to the free drug and its liposomal formulations [187]. Similarly, the PTX loaded EVs decreased the metastatic growth in mouse xenograft model of Lewis lung carcinoma [80]. Further, exosomal delivery of PTX improved its anticancer activity by increasing its solubility and decreasing the Pgp-mediated drug efflux. Another study by Kim et al., 2018, derived the macrophages based EVs to deliver PTX in in-vitro and in-vivo models of metastatic lung carcinoma. They engineered EVs to form AA-PEG-ExoPTX using aminoethylanisamide-polyethylene glycol (AA-PEG) to target the overexpressed sigma receptors in lung cancer. The EVs treatment showed noteworthy suppression of tumor development in-vitro and showed significant decrease in cell viability post treatment [192]. The EVs have also been modified with the introduction of various molecules or proteins on their surface for targeted delivery of the drug. The MSC derived EVs covalently decorated with Mucin-1 (MUC-1) aptamer ensured the efficient transportation of the loaded drug (DOX) to the MUC-1 positive colon cancer cells. The targeted delivery of DOX also resulted in the increased cytotoxic activity of the drug in-vitro and showed around 65% tumor regression in C26 xenograft tumors [193]. Additionally, the naturally derived EVs have also been used to deliver the anticancer therapies in combination to improve their efficacy against resistant cancers. Kumar et al., 2022, used milk EVs functionalized with folic acid, for targeted delivery, loaded with PTX and 5-FU and their combination showed decreased IC_{50} for both exosome loaded drugs (PTX and 5-FU) and their combinations in comparison to the individual drugs [194]. The EVs have also been used to deliver the chemotherapeutic drugs across the brain. Yang et al., 2015, successfully delivered PTX and DOX across the blood brain barrier with improved efficacies in a zebrafish xenotransplanted model of brain cancer [195].

10. EV mediated delivery of cannabinoids

EVs can serve as a potential delivery system for cannabinoids with targeted delivery and enhanced efficacy [59]. The potential anticancer activity of cannabinoids targeting cancer cell proliferation, differentiation, angiogenesis, and metastasis is circumvented by their poor solubility, CYP enzymes induced metabolism and first pass metabolism which limits their bioavailability. The EV loaded delivery of cannabinoids to cancer cells has been investigated by Patel et al., 2021, in which they used the EVs derived from hUCMSCs as a delivery system for CBD which sensitizes the MDA-MB-231 cells to DOX. Briefly, CBD was loaded to the isolated EVs and the EVs were then characterized and optimized, followed by their analysis for antitumor activity. The combination of CBD loaded EVs and DOX significantly decreased the tumor burden in MDA-MD-231 xenografted breast cancer model [201]. The combination also successfully decreased the expression of proteins involved in inflammation, metastasis and showed increase in the expression of apoptotic proteins. The therapeutic potential of EV loaded cannabinoids creates enough room to explore the anticancer efficacy of cannabinoids

in various cancer types alone or in combination with other anticancer agents.

Similarly, EVs can be used to deliver the therapeutic agents to target the toxicities caused by the classical chemotherapeutic agents. The use of cannabinoids in treating cancer related pain and other symptoms, anorexia, anxiety, insomnia, and depression can be explored using the EVs as delivery agents for the same. The delivery of cannabinoids especially for the most common anti-cancer therapy related issue, peripheral neuropathy has been addressed in multiple studies [202]. Among others, paclitaxel induced peripheral neuropathy is one of the most common clinical issues in cancer patients. The efficacy of THCV, CBD and their combination has been evaluated in a study using in-vivo model of PIPN. The neuroprotective effects of cannabinoids were further investigated using RNA sequencing, proteomics and it was observed that there was involvement of genes regulating MAPkinase, PI3K-AKT, AMPK, and inflammasome pathways in PIPN pathogenesis [39].

11. Epigenetic modifications with cannabinoids in cancer

Apart from the primary mechanistic changes at molecular level, epigenetic modifications are also believed to be directly responsible for the development of cancer. Aberrant epigenetic changes have been reported to occur at a very early stage of the neoplastic development. The common epigenetic mechanisms involved in cancer includes: DNA methylation, post translational histone modifications (methylation, acetylation, ubiquitination, phosphorylation, and SUMOylation), chromatin post translational modifications (PTM), as well as non-coding RNAs regulations [203,204]. The histone modifications are the most diverse out of all and histone acetylation usually leads to the active chromatin state while methylation has more diverse and complex effects. Mutations in DNA methyltransferase writer DNMT3A has been reported in acute myelogenous leukemia (AML). Similarly, mutations in TET2, IDH1, IDH2 and DNMT3A has been reported in patients with T cell lymphoma and angioimmunoblastic subtype of peripheral T cell lymphoma (AILT). Also, mutations due to histone in EZH2, NSD2, SETD2, and DOT1L are associated with oncogenesis. The epigenetic targets to treat various cancers have also been widely studied with various therapies targeting the epigenetic regulators such as: DNMTs, KMTs, HDAC inhibitors and therapies targeting writers, erasers and readers [205]. The cannabinoids have not been studied for the epigenetic targets with respect to their anticancer activity but they have been explored for epigenetic changes in suppression of inflammation abuse potential and psychiatric changes for the psychoactive cannabinoids (THC) [206]. In an in-vivo model of staphylococcal Enterotoxin B (SEB) induced lung injury characterized by acute respiratory distress syndrome (ARDS), T cell activation and cytokine storm, the treatment with THC downregulated the expression of miR-17/92 (miR-18a specifically) and miR-374b/421 and upregulated the miR-146a expression. These miRNAs prevent the formation of Tregs (Regulatory T cells) by targeting phosphatase and tensin homologue (Pten) which further inhibits the PI3K/Akt signaling pathway and, therefore THC treatment promotes the generation of Tregs. The upregulation of miR-146a is associated with decrease in the expression of pro-inflammatory cytokines IL-6, IL-12 and IFN-ϒ and induction of cellular apoptosis [207]. Similarly, in another study on the same model of SEB exposed mice, treatment with THC altered the differentiation of T cells from Th1 to Th2 suppressing the histone marks in the Th1-associated genes (H3K9me3 and H3K27me3) and activating the histone marks in Th-2 associated genes (H3K4me3 and H3K36me3 likely) [208].

Kalvala et al., 2023, in their studies observed that H3K4 methylation and H2BK5 acetylation was increased in their DOX resistant MDA-MB-231 tumor lysates and they identified these histone modifications as possible markers of resistance. Whereas, in their study, CBD and THCV treatment in combination with DOX significantly downregulated these modified histones and they linked these alterations with reduced expressions of NF-ϰB, SOD2, Bcl-xl, NLRP3, P-38, PD-L1 and other proteins which are involved in tumor formation and metastasis [39]. Additionally, treatment of mouse lymph node cells with anandamide (endogenous ligand of cannabinoid receptors) differentially regulated miRNAs targeting pro-inflammatory mediators in a delayed-type hypersensitivity model thereby decreasing the pro-inflammatory cytokine production [209]. Paradisi et al., 2008, treated spontaneously immortalized human keratinocytes (HaCaT cells) with endogenous anandamide increased global DNA methylation and resulted in the decreased expression of cell differentiation related genes [210]. Likewise, the anticancer activity of cannabinoids is likely to have epigenetic alterations (gain of function or loss of function) which will further have a considerable potential to address the complex mechanisms and targets to address dire need of cancer therapeutics.

12. Patents on use of EVs for cancer treatment

The extensive research in the field of use of EVs as delivery carriers for anticancer agents have led the researchers to explore various EV based formulations/delivery systems for their efficacy and many patents claiming their delivery systems to have more profound targeted delivery of the anticancer therapeutic agents with increased bioavailability and decreased untoward effects are available [211,212]. Different patents address the issue of delivery of poorly soluble anticancer drugs to the targeted tumors or cancer cells [213]. US10874610B2 patent suggested the role of engineered EVs with loaded proteins targeting peptides in host cancer cells for more profound delivery and also for loading with small molecules [214]. Similarly, CN111840513A patent demonstrated the use of MSC derived EVs containing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and anticancer micromolecules and its role in overcoming the low bioavailability and in-vivo instability of TRAIL and drug resistance [215]. Lexobrid A is a hybrid EV formulation containing EVs loaded lipid conjugated DOX to increase the half-life of the drug, improve its bioavailability and increase the anticancer activity (KR20200145115A) [216]. Patent, KR1020150120934A was originally obtained in Korea_by ILIAS Biologics where the inventors used a novel technique of using optically reversible protein-protein interactions for targeted delivery of soluble proteins as payloads in EVs under their platform named ‘Exosome engineering for Protein Loading via Optically Reversible protein–protein interaction (EXPORE) [217]. Similarly, US10329561 and US10233445 patents demonstrated the EVs (derived from various physiological fluids, Dendritic cells in particular) for the delivery of genetic material including DNA plasmid, miRNA, siRNA, shRNA, modified oligonucleotides to target a variety of diseases [218]. Another patent, US11103586B2 demonstrated the use of EVs loaded with proteins, peptides including antibodies, antibody fragments for targeted delivery. The payload can be transmembrane proteins in EVs allowing the surface expression of the targeted moiety [219]. Approved in 2019, patent KR20200136978A demonstrated the EVs having various growth factors, therapeutic agents and proteins as payload can be used for targeted delivery [220]. In another patent US10617768B2, the innovators engineered the EVs using transmembrane tetraspanins for the delivery of bioactive cargo. Peptides of any form or type can be attached to the various attachment points on the tetraspanin transmembrane anchoring scaffold present in EV membranes making them more biocompatible and illicit strong bioactive cargo loading and delivery in a mammalian system [221]. In yet another patent US10758486B2, the same innovator engineered the EVs using vesicular stomatitis virus glycoprotein (VSVG) by expressing gene-encoding the VSVG fusion proteins in their mother cells. These EVs can be used to deliver functional fluorescent proteins and antibodies to diseased cells for high-contrast imaging and therapy. According to the inventors, pseudotyped EVs with full length VSVG accumulate in multi-recipient cells at a higher rate than VSVG EVs without the ectodomain, confirming that the ectodomain plays a role in cell tropism [221,222]. US20190151456A1 addressed a similar innovation of techniques for creating therapeutic EVs with newly discovered proteins that are

abundant in the EVs lumen. Their procedures entail creating EVs with modified or fragmented EV proteins fused with therapeutic or cargo proteins, or EV proteins at higher concentrations than those seen in natural EVs [223]. Interestingly, patents for liposomal and EV based preparations having cannabinoids as payload targeting range of diseases including inflammatory diseases, neurological disorders, pain have also been filed. Innocan Pharma Corporation filed patent application for their CBD delivery system technology including LTP (liposomal CBD preparation) and CLX (EV based CBD preparation). The inventors believe that their formulation systems have improved pharmacokinetic profile and prolonged the optimum levels of the drug in blood leading to superior clinical outcomes. Their comparison of free CBD given via IV route with CBD delivery systems (LTP and CLX given via subcutaneous route) in-vivo showed exposure of CBD in blood for prolonged periods of time. The therapeutic efficacy and superiority over drug alone (CBD) in terms of efficacy of these preparations in various pathological conditions is yet to be released by the inventors [224]. You may find out what EV technology some research groups are defending using patent data. This could provide information about future features or new characteristics of EVs that they plan to develop.

13. Summary, conclusions and future perspectives

EVs are efficient drug carriers due to their low immunogenicity, safety, and absence of cytotoxicity. Natural EVs have complications in clinical-grade production, consistent isolation, purification, and drug loading making EV-based medication delivery difficult. However, cell-derived lab-made EVs are promising tools in overcoming these obstacles. Also, EVs derived from immune cells have been extensively studied for their anticancer attributes. On the other hand, majority of the research shows that different cannabinoids suppress the development of tumors in-vivo as well as the growth of cancer cells in-vitro, and that the induction of apoptosis is a key factor in the mechanism behind this action. With each cannabinoid, this effect has a different potency and therefore, the varied cannabinoid receptor binding characteristics may have diverse downstream effects. Anticancer activity of cannabinoids is also due to their potentiality in inducing autophagy and increasing intracellular ROS. Early clinical trials using cannabinoids to treat cancer in patients has been conducted, and additional investigations are being planned to determine their effectiveness and safety. If cannabinoids are to be further developed as potential cancer treatments, much more thorough research is required to understand the full potential of the cannabinoids as well as the dose-response relationships and pathways evoked by the various cannabinoids. The combination of EVs and cannabinoids may potentially lead to a game-changing advancement in the treatment of cancer. EVs are constituted of many components that perform a variety of cellular roles despite the fact that their biology is well understood. Inappropriate donor cells may have immunological effects if they are used to isolate EVs which is why selecting the source of EVs is very important factor to be considered. In this review, we emphasized on research that provides support of the cytotoxic activity of cannabinoids, as well as their possible mechanisms of action along with information on cannabinoid loading in EVs and their characterization, and the effectiveness of drug loaded EVs in killing of cancer cells. Despite the significant advancement, there are still numerous hindrances to overcome before EVs may fully acknowledge their capability as drug carriers. To solve the issues and ease future functional research of EVs as carriers of cannabinoids, more investigation is required. With more studies being done on EVs, we are optimistic that the difficulties will be solved quickly, and the clinical translation will be accomplished. For the benefit of many cancer patients, there will be significant advancements achieved in EV-based cancer treatment approaches as EVs are small packages with big potential.

Ethics approval

Not applicable.

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Addressing the reviewers comments

All the comments have been addressed in relevant sections and Comment 5 has also been addressed.

CRediT authorship contribution statement

Sukhmandeep Kaur: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Aakash Nathani:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mandip Singh:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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