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(58) Field of Search:
Other: **WPI, EPODOC, TXTA, BIOSIS, MEDLINE**

(54) Title of the Invention: **CPP-Mediated EV Loading**
Abstract Title: **CPP-mediated extracellular vesicle loading**

(57) A method for loading extracellular vesicles (EVs) with a small molecule diagnostic or therapeutic agents comprising exposing an EV to a small molecule agent and a cell-penetrating peptide (CPP). The CPP and agent may be present in the form of non-covalent complexes or covalent conjugates. Furthermore, the present invention pertains to medical uses and compositions comprising such small molecule-loaded EVs.

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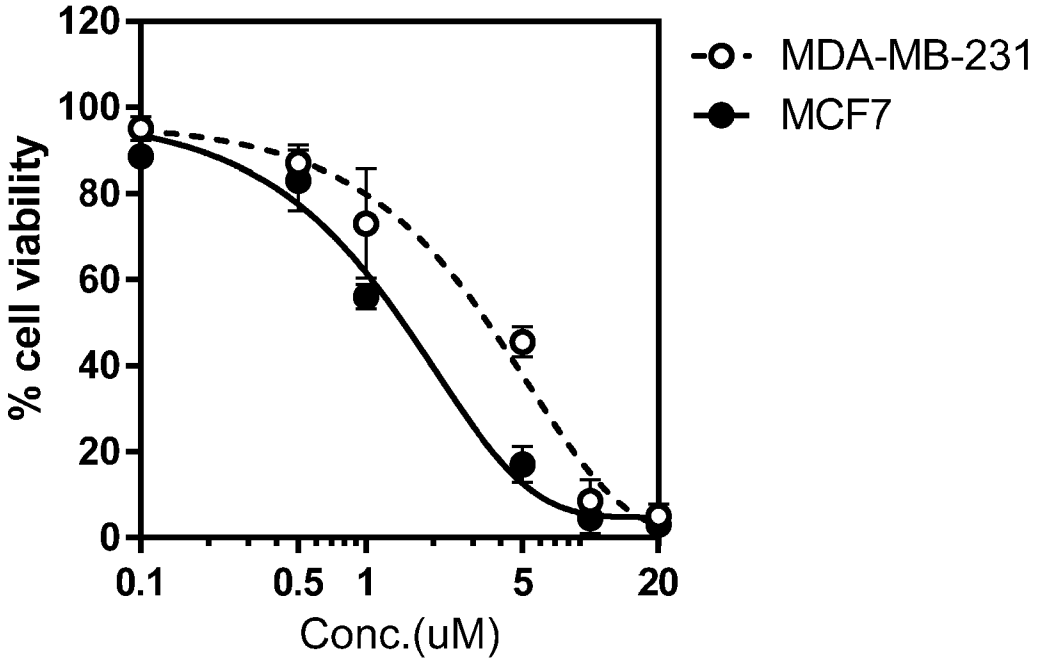
120
100
80
60
40
20
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% cell viability
0.1
0.5
1
5
20
Conc.(uM)
MDA-MB-231
MCF7

Figure 1

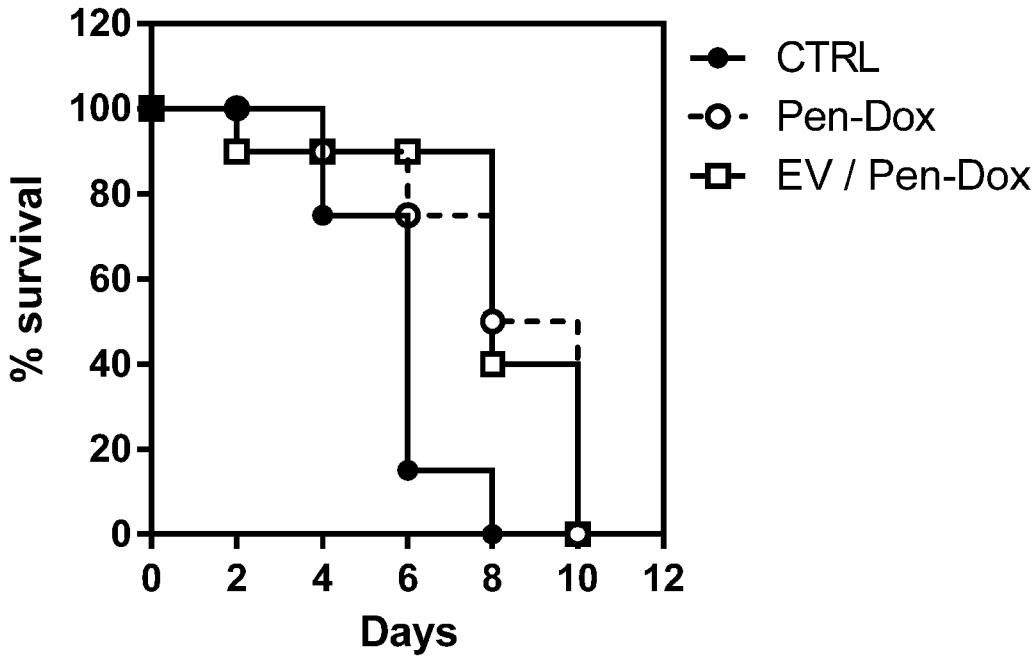
120
100
80
60
40
20
0
% survival
0
2
4
6
8
10
12
Days
CTRL
Pen-Dox
EV / Pen-Dox

Figure 2

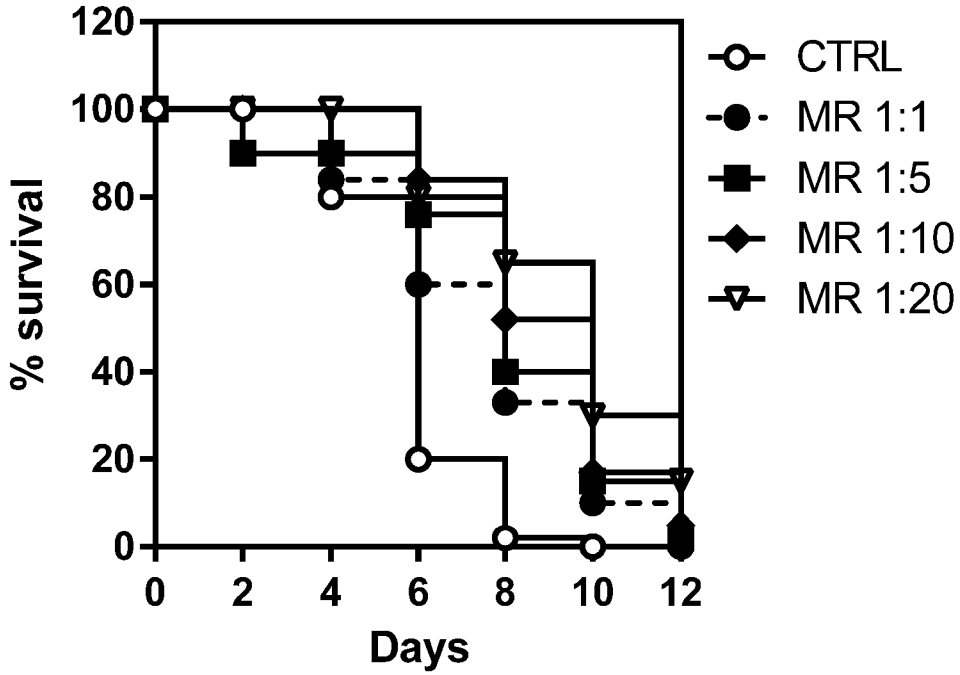
120
100
80
60
40
20
0
% survival
0
2
4
6
8
10
12
Days
CTRL
MR 1:1
MR 1:5
MR 1:10
MR 1:20

Figure 3

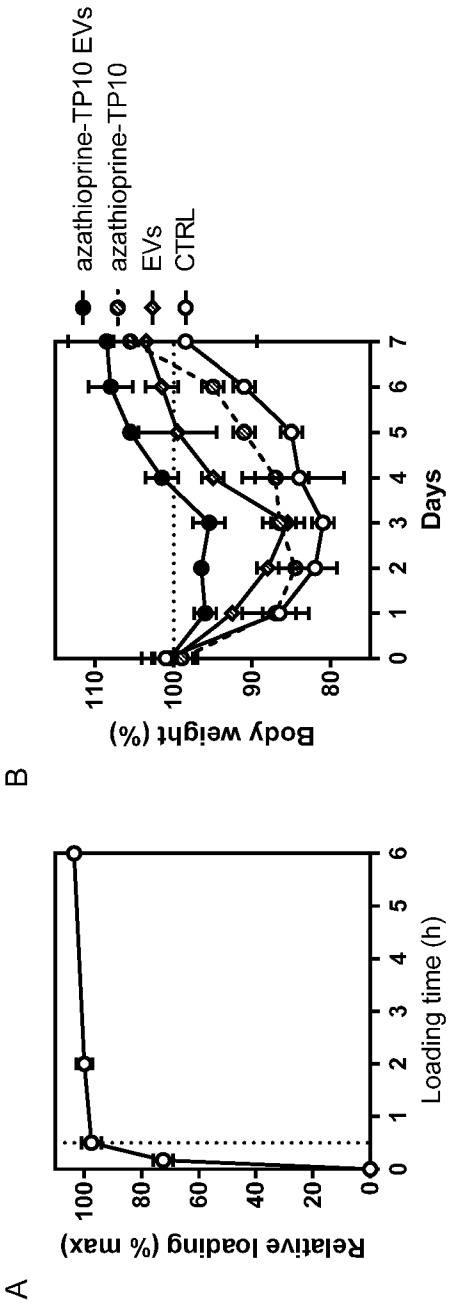
A
Relative loading (% max)
100
80
60
40
20
0
0 1 2 3 4 5 6
Loading time (h)
B
Body weight (%)
110
100
90
80
0 1 2 3 4 5 6 7
Days
azathioprine-TP10 EVs
azathioprine-TP10
EVs
CTRL

Figure 4

CPP-mediated EV loading

Technical field

The present invention relates to methods for loading extracellular vesicles (EVs) with a small molecule diagnostic or therapeutic agent, comprising exposing a population of EVs to either a conjugate or a non-covalent complex or a combination thereof comprising at least one small molecule agent and at least one cell-penetrating peptide (CPP).

Background art

Extracellular vesicles (EVs) modulate cell-to-cell communication in normal physiology and pathology by presenting their contents (primarily RNA, proteins, and lipids) to recipient cells in target tissues. Modification of EVs to incorporate various types of pharmacological agents have been explored in numerous contexts, for instance WO2013/084000, which discloses the use of exosomes for intracellular delivery of biotherapeutics, or WO2010/119256, which describes delivery of exogenous genetic material using exosomes.

The utility of EVs as drug delivery vehicles is unquestionable in the case of for instance nucleic acid based drugs such as siRNA, large protein-based drugs targeting intracellular components, and e.g. poorly soluble or highly toxic small molecule therapeutic agents. EV-mediated small molecule drug delivery has also been explored to a great extent, with for instance WO2011/097480 representing the typical approach to loading of EVs. WO2011/097480 describes a very facile method wherein e.g. the phytochemical small molecule agents curcumin and resveratrol are loaded into EVs using a simple co-incubation step during which purified EVs and free drug (e.g. curcumin) are allowed to incubate together in phosphate buffered saline (PBS) at room temperature, relying on diffusion of the drug into the EV. Although highly convenient and straightforward, this conventional approach to loading small molecule agents into EVs is not particularly efficient, results in significant waste of the small molecule, and is also very difficult to control. Others (for instance Fuhrman *et al*, J. Control Rel., 2015) have also evaluated permeabilization of EVs, using

detergents such as saponin, as a way of increasing the loading efficiency of in this case the photoactive agent porphyrin.

A recent patent application (WO2015/120150) is also concerned with loading of tumor-derived EVs with various types of anticancer drugs, covering both small molecules and large biopharmaceuticals. However, as is often the case in the art, very little information is available on how to load exosomes and if there are methods available they are rarely useful for loading and actual therapeutic application of small molecule-carrying EVs.

Summary of the invention

It is hence an object of the present invention to overcome the above-identified problems associated with the loading of small molecule agents (typically small molecule drugs or diagnostic agents) into EVs for subsequent therapeutic application. Furthermore, the present invention aims to satisfy other existing needs within the art, for instance to enable loading of significant amounts of small molecule drugs into EVs, to enable controllable loading, and to provide small molecule-loaded EVs with considerable therapeutic potential.

The present invention achieves these and other objectives by utilizing cell-penetrating peptides (CPPs) as a carrier of small molecule agents into EVs. In one aspect, the present invention relates to methods for EV loading comprising the step of exposing a population of EVs to at least one small molecule agent and at least one CPP. The small molecule and the CPP may be covalently conjugated into one molecule, with one CPP carrying at least one small molecule agent. Alternatively, the CPP and the small molecule agent may also form nanoparticle complexes as a result of non-covalent interactions, which may be surmised to be e.g. hydrophobic and/or electrostatic and/or van der Waals interactions. Interestingly, although CPP-small molecule conjugates may be present in the form of individual conjugates they may also form non-covalent nanoparticle complexes.

In a further aspect, the present invention pertains to methods of loading EVs with small molecule agents through loading of the EV source cells. Such methods may comprise the steps of (a) exposing a population of EV source cells to at least one

small molecule agent and at least one CPP and (b) harvesting EVs produced by the EV source cells, wherein the EVs comprise the small molecule agent(s) in question.

In another aspect, the present invention pertains to EVs comprising at least one small molecule agent conjugated to or complexed with at least one CPP, and also to EVs within which a small molecule agent has been released from a CPP conjugate and/or a CPP nanoparticle complex. The small molecule agents of the present invention may be selected from a wide variety of drug or diagnostic agent categories, for instance anticancer agents such as doxorubicin, 5-fluorouracil or other nucleoside analogues such as cytosine arabinoside, proteasome inhibitors such as bortezomib, or kinase inhibitors such as imatinib or seliciclib, or NSAIDs such as naproxen, aspirin, or celecoxib, antibiotics such as heracillin, or antihypertensives such as ACE inhibitors such as enalapril, ARBs such as candesartan, etc.

In yet another aspect, the present invention pertains to methods for delivering small molecule drugs to a target cell, a target tissue, a target organ, or to any target compartment (which may also include bodily fluids, for instance the blood stream or cerebrospinal fluid). Such methods may comprise exposing the target to EV loaded with a small molecule using the CPP-mediated strategies of the present invention.

In a further aspect, the present invention also relates to methods of altering the pharmacokinetic or pharmacodynamics profile of a small molecule drug. Such methods involve comprise CPP-mediated loading of the small molecule in question into an EV, in order to modulate *in vivo* and potentially also *in vitro* properties of the small molecule drug in question.

Additionally, in further aspects, the present invention pertains to pharmaceutical compositions comprising small molecule-carrying EVs as per the present invention, or in practical terms compositions comprising populations of small molecule-carrying EVs as per the present invention. The EV concentration in such compositions may be expressed in many different ways, for instance amount of EV protein per unit (often volume) or per dose, number of particles per unit (often volume) or per dose, concentration of small molecule drug per unit or per dose, etc. Typically, such pharmaceutical compositions are formulated for in use *in vivo* and also *in vitro* using pharmaceutically acceptable excipients.

Finally, the present invention also relates to medical uses and applications of small molecule-carrying EVs, for instance in the treatment of inflammatory diseases, autoimmune diseases, cancer, metabolic disorders, or any suitable disease or disorder.

Brief description of the drawings

Figure 1 illustrates *in vitro* data on immune cell-derived EVs loaded with penetratin-doxorubicin conjugates, evaluated using the MTT assay.

Figure 2 shows *in vivo* evaluation in mice of penetratin-doxorubicin conjugates loaded into immune cell EVs.

Figure 3 shows *in vivo* evaluation of DC-EVs loaded with CADY1-paclitaxel non-covalent complexes, using the same mice model as in Figure 2.

Figure 4 illustrates the anti-inflammatory effects *in vivo* of MSC-EVs loaded with stearylated TP10-azathioprine complexes in TNBS-induced colitis.

Detailed description of the invention

The present invention describes *inter alia* novel methods, compositions, and uses of EVs for the delivery of small molecules. More specifically, the present invention relates to methods for EV loading, EVs loaded with small molecules, various methods for utilizing such EVs, pharmaceutical compositions comprising EVs in therapeutically effective amounts, and medical uses of small molecule-loaded EVs as per the present invention.

For convenience and clarity, certain terms employed herein are collected and described below. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

Where features, aspects, embodiments, or alternatives of the present invention are described in terms of Markush groups, a person skilled in the art will recognize that the invention is also thereby described in terms of any individual member or

subgroup of members of the Markush group. The person skilled in the art will further recognize that the invention is also thereby described in terms of any combination of individual members or subgroups of members of Markush groups. Additionally, it should be noted that embodiments and features described in connection with one of the aspects and/or embodiments of the present invention also apply mutatis mutandis to all the other aspects and/or embodiments of the invention. For example, the various small molecules described in connection with the methods for CPP-mediated loading of EVs are to be understood to be disclosed, relevant and included also in the context of the pharmaceutical compositions comprising small molecule-carrying EVs. Furthermore, certain embodiments described in connection with certain aspects, for instance the administration routes of the small molecule-loaded EVs, as described in relation to aspects pertaining to treating certain medical indications with EVs as such, may naturally also be relevant in connection with other aspects and/or embodiment such as those pertaining to the pharmaceutical compositions of the present invention. Moreover, any and all features (for instance any and all members of a Markush group) can be freely combined with any and all other features (for instance any and all members of any other Markush group), e.g. any EV protein may be combined with any targeting moiety, or any EV cell source may be combined with any small molecule agent. Furthermore, when teachings herein refer to EVs in singular and/or to EVs as discrete natural nanoparticle-like vesicles it should be understood that all such teachings are equally relevant for and applicable to a plurality of EVs and populations of EVs. As a general remark, the small molecules, the CPPs, the targeting moieties, the cell sources, the exosomal proteins, and all other aspects, embodiments, and alternatives in accordance with the present invention may be freely combined in any and all possible combinations without deviating from the scope and the gist of the invention. Furthermore, any polypeptide or polynucleotide or any polypeptide or polynucleotide sequences (amino acid sequences or nucleotide sequences, respectively) of the present invention may deviate considerably from the original polypeptides, polynucleotides and sequences as long as any given molecule retains the ability to carry out the technical effect associated therewith. As long as their biological properties are retained the polypeptide and/or polynucleotide sequences according to the present application may deviate with as much as 50% (calculated using for instance BLAST or ClustalW) as compared to the native sequence, although a sequence identity that is as high as

possible is preferable (for instance 60%, 70%, 80%, or e.g. 90% or higher). The combination (fusion) of e.g. at least one targeting polypeptide and at least one exosomal protein implies that certain segments of the respective polypeptides may be replaced and/or modified, meaning that the deviation from the native sequence may be considerable as long as the key properties (such as the targeting properties and trafficking to the surface of exosomes in this particular case) are conserved. Similar reasoning thus naturally applies to the polynucleotide sequences encoding for such polypeptides.

The terms "extracellular vesicle" or "EV" or "exosome" are used interchangeably herein and shall be understood to relate to any type of vesicle that is obtainable from a cell in any form, for instance a microvesicle (e.g. any vesicle shed from the plasma membrane of a cell), an exosome (e.g. any vesicle derived from the endo-lysosomal pathway), an apoptotic body (e.g. obtainable from apoptotic cells), a microparticle (which may be derived from e.g. platelets), an ectosome (derivable from e.g. neutrophils and monocytes in serum), prostatosome (e.g. obtainable from prostate cancer cells), or a cardiosome (e.g. derivable from cardiac cells), etc. Furthermore, the said terms shall also be understood to relate to lipoprotein particles, such as LDL, VLDL, HDL and chylomicrons, as well as liposomes, extracellular vesicle mimics, cell membrane-based vesicles obtained through membrane extrusion or other techniques, etc. Essentially, the present invention may relate to any type of lipid-based structure (with vesicular morphology or with any other type of suitable morphology) that can act as a delivery or transport vehicle for small molecules of interest. It will be clear to the skilled artisan that when describing medical and scientific uses and applications of the EVs, the present invention normally relates to a plurality of EVs, i.e. a population of EVs which may comprise thousands, millions, billions or even trillions of EVs. As can be seen from the experimental section below, EVs may be present in concentrations such as 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14} or similar per ml. In the same vein, the term "population", which may e.g. relate to an EV comprising a certain small molecule and often a certain CPP, shall be understood to encompass a plurality of entities constituting such a population. In other words, individual EVs when present in a plurality constitute an EV population. Thus, naturally, the present invention pertains both to individual EVs comprising small molecules and populations comprising EVs comprising small molecules, as will be

clear to the skilled person. The dosages of EVs when applied *in vivo* may naturally vary considerably depending on the disease to be treated, the administration route, the small molecule cargo, etc.

The term "small molecule agent" or "small molecule" or "small molecule drug" or "small molecule therapeutic" are used interchangeably herein and shall be understood to relate to any molecular agent which may be used for the treatment and/or diagnosis of a disease and/or disorder. Small molecule agents are normally synthesized via chemical synthesis means, but may also be naturally derived, for instance via purification from natural sources, or may be obtained through any other suitable means or combination of techniques. A brief definition of a "small molecule" is any organic compound with a molecular weight of less than 900 g/mol (Dalton) that may help to regulate a biological process. For the purposes of this invention, small molecule may be substantially larger than 900 g/mol, for instance 1500 g/mol, 3000 g/mol, or occasionally even larger. Although many small molecules exhibit good oral bioavailability many small molecule drugs need to be given intravenously or via some other route of administration, be it for pharmacokinetic, pharmacodynamics, and/or toxicity or stability reasons. Examples of small molecules include anticancer agents such as doxorubicin, 5-fluorouracil, proteasome inhibitors such as bortezomib, or kinase inhibitors such as imatinib or seliciclib, or NSAIDs such as naproxen, aspirin, or celecoxib, antibiotics such as heracillin, or antihypertensives such as ACE inhibitors such as enalapril, ARBs such as candesartan, etc. The present invention is naturally applicable also to other small molecules without departing from the gist of the invention, as would be clear to a person skilled in the art.

The terms "cell-penetrating peptide" and "CPP" are used interchangeably herein and shall be understood to relate to relatively short peptides (≤50 amino acids) with the ability to gain access to the interior of virtually any cell type. CPPs are typically highly cationic and rich in arginine and lysine amino acids. They have the exceptional property of being able to carry into cells and/or EVs a wide variety of covalently (conjugated) and/or non-covalently attached cargoes such as proteins, oligonucleotides, and as in the present invention small molecule drugs. CPPs as per the present invention includes, but are not limited to, transportan, transportan 10, penetratin, MTS, VP22, CADY peptides, MAP, KALA, PpTG20, proline-rich peptides, MPG peptides, PepFect peptides, Pep-1, L-oligomers, calcitonin-peptides, arginine-

rich CPPs such as poly-Arg, Tat, and combinations thereof. CPPs in accordance with the present invention also includes similar classes of peptides such as antimicrobial peptides, membrane-active peptides, and peptidic ligands to receptors that are already present on EV membrane which may facilitate internalization or interaction with an EV. Furthermore, various types of chemical modifications have been introduced on CPPs with great success, and the CPPs of the present invention may thus be modified through the introduction of for instance lipid tails, cholesterol and cholesterol analogues, quinolones and specifically chloroquine and its fluorinated analogues, and other types of C- and/or N-terminal and/or orthogonal modifications. These types of chemical modifications may for example improve small molecule complexation, internalization into EVs or interaction with the EV surface, in addition to modulating size and zeta potential. In addition to chemical modifications being made to CPPs *per se*, the CPPs per the present invention may include synthetic and/or artificial peptide derivatives, for instance so called peptidoids which includes CPPs containing non-natural amino acids, inverso and/or retro-inverso analogues, modifications from L- to D-amino acids, peptides containing residues that mimic protein post-translational modifications, and other types of desirable modifications known to the person skilled in the art. Furthermore, as is clear in the context of CPP-small molecule conjugates, CPPs may be functionalized to comprise moieties that enable chemical conjugation or even complexation with the small molecule cargo.

The terms “EV protein” and “EV polypeptide” “exosomal polypeptide” and “exosomal protein” are used interchangeably herein and shall be understood to relate to any polypeptide that can be utilized to transport a polypeptide construct (which typically comprises, in addition to the exosomal protein, a targeting peptide or polypeptide) to a suitable vesicular structure, i.e. to a suitable EV. More specifically, the term “exosomal polypeptide” shall be understood as comprising any polypeptide that enables transporting, trafficking or shuttling of a polypeptide construct (which as abovementioned typically comprises at least one targeting peptide/polypeptide) to a vesicular structure, such as an exosome. Examples of such exosomal polypeptides are for instance CD81, CD9, Itab1, Mfge8, CD63, CD151, Hspg2, Lgals3bp, Col6a1, Agrn, Tspam14, Lamc1, Lamb1, Tfrc, CD47, CD82, Slit2, Syntenin, Alix, Syndecan, and Lamp2, Lamp2b, CD13, CD86, Flotillin, Syntaxin-3, CD2, CD36, CD40, CD40L, CD41a, CD44, CD45, ICAM-1, Integrin alpha4, LiCAM, LFA-1, Mac-1 alpha and beta,

Vti-1A and B, CD3 epsilon and zeta, CD18, CD37, CD53, CD82, CXCR4, FcR, GluR2/3, HLA-DM, immunoglobulins, MHC-I or MHC-II components, TCR beta, and tetraspanins generally, but numerous other polypeptides capable of transporting a polypeptide construct to an EV are comprised within the scope of the present invention.

The terms "source cell" or "EV source cell" or "parental cell" or "cell source" or "EV-producing cell" or any other similar terminology shall be understood to relate to any type of cell that is capable of producing EVs under suitable cell culturing conditions, for instance in suspension culture or in adherent culture or any in other type of culturing system. The source cells per the present invention may be select from a wide range of cells, for instance mesenchymal stem or stromal cells (obtainable from e.g. bone marrow, adipose tissue, Wharton's jelly, perinatal tissue, tooth buds, umbilical cord blood, etc.), amnion cells, myeloid suppressor cells, immortalized cell lines of which human embryonic kidney (HEK) cells represent one non-limiting example, dendritic cells (DCs) or other immune system cells such as macrophages (e.g. M2 polarized macrophages), monocytes, B- or T-cells, NK cells, neutrophils, eosinophils, mast cells or basophils, etc. Generally, EVs may be derived from essentially any cell source, be it a primary cell source or an immortalized cell line. The EV source cells may be any embryonic, fetal, and adult somatic stem cell types, including induced pluripotent stem cells (iPSCs) and other stem cells derived by any method. When treating neurological diseases, one may contemplate to utilize as source cells e.g. primary neurons, astrocytes, oligodendrocytes, microglia, and neural progenitor cells. The source cell may be either allogeneic, autologous, or even xenogeneic in nature to the patient to be treated, i.e. the cells may be from the patient himself or from an unrelated, matched or unmatched donor. In certain contexts, allogeneic cells may be preferable from a medical standpoint, as they could provide immuno-modulatory effects that may not be obtainable from autologous cells of a patient suffering from a certain indication. For instance, in the context of treating systemic, peripheral and/or neurological inflammation, allogeneic MSCs may be preferable as EVs obtainable from such cells may enable immuno-modulation via e.g. macrophage and/or neutrophil phenotypic switching (from pro-inflammatory M1 or N1 phenotypes to anti-inflammatory M2 or N2 phenotypes, respectively). Conversely,

when utilizing EVs for treating a solid or hematological malignancy, it may be preferable to select immune cells such as DCs as the EV-producing cell source.

In a first aspect, the present invention relates to methods for loading EVs with small molecule agents, wherein the methods comprise exposing a population of EVs to at least one small molecule agent and at least one CPP. CPPs, which normally comprise a mix of cationic and hydrophobic amino acid residues, are either covalently conjugated to or non-covalently complexed with the small molecule cargo. Non-covalent complexes between CPPs and the small molecule cargo normally form nanoparticle-like structures which can be studied and characterized with conventional techniques for nanoparticle research, for instance dynamic light scattering and nanoparticle tracking analysis. Conjugates between small molecules and CPPs occasionally also form nanoparticles, due to e.g. electrostatic and/or hydrophobic interactions between individual conjugates leading to aggregation into supramolecular structures. Regardless of the whether the complexes comprise non-covalent or conjugated small molecules and CPPs such complexes may display certain nanoparticle-like features, such as size and zeta potential. The zeta potential of the complexes of present invention may vary substantially depending on the CPP and the small molecule in question, but advantageously the zeta potential is either positive or negative with an absolute value of preferably +/- 10 mV, more preferably +/- 20 mV, or even more preferably +/- 30 mV, or even higher/lower, in order to display colloidal stability. The sizes of such nanoparticle complexes may also vary considerably but in order to enable efficient incorporation into EVs it is preferably if the size of the complex is smaller than the size of the EV. As an example, an EV of a size of 120 nm would preferably be loaded with a small molecule in the form of a CPP-containing nanoparticle having a size well below 120 nm, e.g. 10 nm.

In a further aspect, the present invention pertains to methods of loading EVs with small molecule agents through loading of the EV source cells. This approach is especially advantageous when using CPPs that are known to internalize into cellular compartments associated with production/secretion of EVs, for instance the endo-lysosomal pathways and the plasma membrane. Such methods may comprise the steps of (a) exposing a population of EV source cells to at least one small molecule agent and at least one CPP and (b) harvesting EVs produced by the EV source cells, wherein the EVs comprise the small molecule agent(s) in question.

As above-mentioned, in certain aspects of the invention the at least one small molecule agent and the at least one CPP may be present in the form or covalent conjugates, non-covalent complexes or a combination thereof. There are multiple strategies available for covalent conjugation/linkage, for instance the formation between a CPP and a small molecule of any type of chemical bond selected for instance from the group comprising an ester bond, an amide bond, a disulfide bond, a thioether bond, a biotin-streptavidin interaction, a linkage obtained through a maleimide-NHS reaction, a linkage obtained through a EDC-NHS reaction, a stapled linkage (for instance an all-hydrocarbon staple) and various other conjugation techniques.

In yet another embodiment, the loading of the CPP-small molecule complexes and/or conjugates may be enhanced through the use of a transfection reagent, such as a liposome and/or a lipid nanoparticle. By combining CPPs and a transfection reagent one can in certain instances improve the small molecule loading into EVs and into the EV source cells if that method is utilized.

In yet another aspect, the present invention relates to an EV comprising at least one small molecule drug conjugated to and/or complexed with at least one CPP. Furthermore, the present invention also pertains to EVs within which the at least one small molecule agent has been released from the at least one CPP conjugate and/or CPP complex inside the EV. The terms “inside the EV” and “in the EV” shall be understood to comprise the EV in its entirety, including the membrane of the EV, or even onto the external surface of the EV, as long as the small molecule is in some way interacting with the EV and wherein the EVs are carrying, in any way, the small molecule agent(s) *per se*.

As abovementioned, the small molecule drugs as per the present invention can be obtained from essentially the entire space of pharmaceutically and/or pharmacologically and/or diagnostically relevant agents, for instance anticancer agents, cytostatic agents, tyrosine kinase inhibitors, statins, NSAIDs, antibiotics, antifungal agents, antibacterial agents, anti-inflammatory agents, anti-fibrotics, antihypertensives, aromatase or esterase inhibitors, an anticholinergics, SSRIs, BKT inhibitors, PPAR agonists, HER inhibitors, AKT inhibitors, BCR-ABL inhibitors, signal transduction inhibitors, angiogenesis inhibitors, synthase inhibitors, ALK inhibitors, BRAF inhibitors, MEK inhibitors, PI3K inhibitors, neprilysin inhibitors, beta2-agonists,

CRTH2 antagonists, FXR agonists, BACE inhibitors, sphingosine-1-phosphate receptor modulators, MAPK inhibitors, Hedgehog signaling inhibitors, MDM2 antagonists, LSD1 inhibitors, lactamase inhibitors, TLR agonists, TLR antagonists, IDO inhibitors, ERK inhibitors, Chk1 inhibitors, splicing modulatory, DNA or RNA intercalators, etc. Other non-limiting examples of small molecule drugs as per the present invention includes for instance everolimus, trabectedin, abraxane, pazopanib, enzastaurin, vandetanib, a FLT-3 inhibitor, a VEGFR inhibitor, an EGFR TK inhibitor, an aurora kinase inhibitor, a PIK-1 modulator, a Bcl-2 inhibitor, an HDAC inhbitor, a c-MET inhibitor, a PARP inhibitor, a Cdk inhibitor, an EGFR TK inhibitor, an IGFR-TK inhibitor, an anti-HGF antibody, a PI3 kinase inhibitors, an AKT inhibitor, a JAK/STAT inhibitor, a checkpoint-1 or 2 inhibitor, a focal adhesion kinase inhibitor, a Map kinase kinase (mek) inhibitor, pemetrexed, erlotinib, dasatanib, nilotinib, decatanib, panitumumab, amrubicin, oregovomab, nolatrexed, batabulin, ofatumumab, zanolimumab, edotecarin, tetrandrine, rubitecan, tesmilifene, oblimersen, ticilimumab, ipilimumab, gossypol, cilengitide, gimatecan, lucanthone, neuradiab, vitespan, talampanel, atrasentan, romidepsin, sunitinib, 5-fluorouracil, vorinostat, etoposide, gemcitabine, doxorubicin, 5'-deoxy-5-fluorouridine, vincristine, temozolomide, seliciclib, capecitabine, camptothecin, PEG-labeled irinotecan, tamoxifen, toremifene citrate, anastrazole, exemestane, letrozole, vatalanib, goserelin acetate, leuprolide acetate, triptorelin pamoate, medroxyprogesterone acetate, hydroxyprogesterone caproate, megestrol acetate, raloxifene, bicalutamide, flutamide, nilutamide, megestrol acetate, erlotinib, lapatanib, canertinib, lonafarnib, tipifarnib, amifostine, suberoyl analide hydroxamic acid, valproic acid, trichostatin sorafenib, arnsacrine, anagrelide, bleomycin, buserelin, busulfan, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, diethylstilbestrol, epirubicin, fludarabine, fludrocortisone, fluoxymesterone, flutamide, gemcitabine, hydroxyurea, idarubicin, ifosfamide, imatinib, leuprolide, levamisole, lomustine, mechlorethamine, melphalan, 6-mercaptopurine, mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, octreotide, oxaliplatin, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, teniposide, testosterone, thalidomide, thioguanine, thiotepa, tretinoin, vindesine, 13-cis-retinoic acid, phenylalanine mustard, uracil mustard, estramustine, altretamine, floxuridine, 5-deooxyuridine, cytosine arabinoside, 6-mecaptopurine, deoxycoformycin, calcitriol,

valrubicin, mithramycin, vinblastine, vinorelbine, topotecan, razoxin, marimastat, COL-3, neovastat, squalamine, endostatin, vitaxin, droloxifene, idoxyfene, spironolactone, finasteride, cimitidine, trastuzumab, denileukin diftitox,gefitinib, bortezimib, paclitaxel, cremophor-free paclitaxel, docetaxel, epithilone B, droloxifene, 4-hydroxytamoxifen, pipendoxifene, arzoxifene, fulvestrant, acolbifene, lasofoxifene, idoxifene, topotecan, rapamycin, temsirolimus, zolendronate, prednisone, lenalidomide, gemtuzumab, hydrocortisone, dexrazoxane, alemtuzumab, all-transretinoic acid, ketoconazole, megestrol, immune globulin, nitrogen mustard,methylprednisolone, ibritgumomab tiuxetan, androgens, decitabine, hexamethylmelamine, bexarotene, tositumomab, arsenic trioxide, cortisone, editronate, mitotane, cyclosporine, liposomal daunorubicin, Edwina-asparaginase, strontium 89, casopitant, netupitant, an NK-1 receptor antagonists, palonosetron, aprepitant, diphenhydramine, hydroxyzine, metoclopramide, lorazepam, alprazolam, haloperidol, droperidol, dronabinol, dexamethasone, methylprednisolone, prochlorperazine, granisetron, ondansetron, dolasetron, tropisetron, pegfilgrastim, erythropoietin, epoetin alfa and darbepoetin alfa, efavirinz among others. As mentioned above, the present invention is naturally applicable also to other small molecules without departing from the gist of the invention, as would be clear to a person skilled in the art.

In a further embodiment, the CPPs for loading of small molecules into EVs and/or EV source cells may be selected from the group comprising transportan, transportan 10, penetratin, CADY peptides such as CADY-1, MTS, VP22, MAP, KALA, PpTG20, prolin-rich peptides, MPG peptides, PepFect peptides, Pep-1, L-oligomers, calcitonin-peptides, arginine-rich CPPs such as poly-Arg, Tat, or any modified and/or synthetic peptides or peptide derivatives thereof. Chemical modifications of CPPs of particular utility are fatty acid modifications, such as covalent attachment of a lipid tail e.g. in the form of stearic acid, or any other type of lipid modification, such as the addition of a cholesterol moiety. Lipid modifications of particular relevance for complexation, internalization and overall efficacy in terms of EV loading include fatty acids comprising 10-30 carbon chosen from stearic acid, lauric acid, myristic acid, palmitic acid, arachidic acid, and behenic acid, or any derivatives thereof, especially un-saturated fatty acid derivative thereof. Chemical modifications of CPPs may further include one or more moieties covalently linked to the N-terminus of the peptide, the

C-terminus of the peptide, or orthogonally anywhere along the peptide. These one or more moieties may be selected from a diverse range of chemical groups, such as acetyl groups, a stearyl groups, cholesteryl, a quinoline such as chloroquine or modified versions thereof, a poly-ethylene glycol, a nuclear localization signal, a nuclear export signal, an antibody or antibody fragment thereof, a peptide, a polysaccharide, a targeting molecule, cysteamide group, a cysteine, a thiol, an amide, a nitrilotriacetic acid, a carboxyl group, a linear or branched alkyl group, a primary or secondary amine, an osidic derivative, a lipid, a phospholipid, a fatty acid, a cholesterol, a poly-ethylene glycol, etc.

In yet another embodiment, the EVs as per the present invention may comprise at least one targeting moiety displayed on the surface of the EV, to even further enhance its therapeutic potential by targeting a tissue, an organ, or cell type of interest. The targeting moiety normally comprises a sequence of amino acids, which may be identified for instance through phage display or any other type of screening methodology. The targeting moiety is typically displayed on the EV surface through genetic engineering of the EV source cells, wherein the source cells are transfected to produce EVs comprising a fusion protein comprising the targeting moiety and an exosome protein. Exosome proteins as per the present invention includes, *inter alia*, CD81, CD9, Itab1, Mfge8, CD63, CD151, Hspg2, Lgals3bp, Col6a1, Agrn, Tspam14, Lamc1, Lamb1, Tfrc, CD47, CD82, Slit2, Syntenin, Alix, Syndecan, and Lamp2, Lamp2b, CD13, CD86, Flotillin, Syntaxin-3, CD2, CD36, CD40, CD40L, CD41a, CD44, CD45, ICAM-1, Integrin alpha4, LiCAM, LFA-1, Mac-1 alpha and beta, Vti-1A and B, CD3 epsilon and zeta, CD18, CD37, CD53, CD82, CXCR4, FcR, GluR2/3, HLA-DM, immunoglobulins, MHC-I or MHC-II components, TCR beta, and tetraspanins.

In a further aspect, the present invention relates to a method of delivering a small molecule drug to a target cell. Such delivery methods may comprise exposing a target cell, or a target tissue or target organ (which may include fluids and liquids such as blood, interstitial fluid, cerebrospinal fluid, etc.), to an EV as per the present invention. As above-mentioned, the EVs may comprise a targeting moiety expressed on its surface, or it may rely on natural tropism and targeting, or it may be non-targeted. Delivery to a target cell can be carried out *in vitro* and/or *in vivo*, depending on the context. Further, the present invention pertains to a method of altering the

pharmacokinetic or pharmacodynamics profile of a small molecule drug. This can be achieved through loading the small molecule agent in question into an EV, which will naturally affect factors such as distribution, enzymatic activity, tissue penetration, etc.

In yet another aspect, the present invention pertains to pharmaceutical compositions comprising EVs comprising small molecule drugs conjugated to or complexed with CPPs. Typically, the pharmaceutical compositions as per the present invention comprise one type of therapeutic EV (i.e. a population of EVs comprising a certain desired small molecule(s)) formulated with at least one pharmaceutically acceptable excipient, but more than one type of EV population may be comprised in a pharmaceutical composition, for instance in cases where a combinatorial treatment is desirable. The at least one pharmaceutically acceptable excipient may be selected from the group comprising any pharmaceutically acceptable material, composition or vehicle, for instance a solid or liquid filler, a diluent, an excipient, a carrier, a solvent or an encapsulating material, which may be involved in e.g. suspending, maintaining the activity of or carrying or transporting the EV population from one organ, or portion of the body, to another organ, or portion of the body (e.g. from the blood to any tissue and/or organ and/or body part of interest).

The present invention also relates to cosmetic and dermatological applications of small molecule-carrying EVs. Thus, the present invention pertains to skin care products such as creams, lotions, gels, emulsions, ointments, pastes, powders, liniments, sunscreens, shampoos, etc., comprising a suitable EV, in order to improve and/or alleviate symptoms and problems such as dry skin, wrinkles, folds, ridges, and/or skin creases. In one embodiment, EVs (which comprise a small molecule of interest) are obtained from a suitable EV-producing cell source with regenerative properties (for instance a mesenchymal stem cell) are comprised in a cosmetic cream, lotion, or gel for use in the cosmetic or therapeutic alleviation of wrinkles, lines, folds, ridges and/or skin creases.

In yet another aspect, the present invention relates to EVs as per the present invention for use in medicine. Naturally, when an EV comprising a small molecule (conjugated and/or complexed with a CPP) in accordance with the present invention is used in medicine, it is in fact normally a population of EVs that is being used. The dose of EVs administered to a patient will depend on the amount small molecule drug

that has been loaded into the EV, the disease or the symptoms to be treated or alleviated, the administration route, the pharmacological action of the small molecule itself, the inherent properties of the EV, as well as various other parameters of relevance.

The EVs and the EV populations as per the present invention may thus be used for prophylactic and/or therapeutic purposes, e.g. for use in the prophylaxis and/or treatment and/or alleviation of various diseases and disorders. A non-limiting sample of diseases wherein the EVs as per the present invention may be applied comprises Crohn's disease, ulcerative colitis, ankylosing spondylitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, sarcoidosis, idiopathic pulmonary fibrosis, psoriasis, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the interleukin-1 receptor antagonist (DIRA), endometriosis, autoimmune hepatitis, scleroderma, myositis, stroke, acute spinal cord injury, vasculitis, Guillain-Barré syndrome, acute myocardial infarction, ARDS, sepsis, meningitis, encephalitis, liver failure, kidney failure, heart failure or any acute or chronic organ failure and the associated underlying etiology, graft-vs-host disease, Duchenne muscular dystrophy and other muscular dystrophies, lysosomal storage diseases such as Gaucher disease, Fabry's disease, MPS I, II (Hunter syndrome), and III, Niemann-Pick disease, Pompe disease, etc., neurodegenerative diseases including Alzheimer´s disease, Parkinson´s disease, Huntington's disease and other trinucleotide repeat-related diseases, dementia, ALS, cancer-induced cachexia, anorexia, diabetes mellitus type 2, and various cancers. Virtually all types of cancer are relevant disease targets for the present invention, for instance, Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia, Adrenocortical carcinoma, AIDS-related cancers, AIDS-related lymphoma, Anal cancer, Appendix cancer, Astrocytoma, cerebellar or cerebral, Basal-cell carcinoma, Bile duct cancer, Bladder cancer, Bone tumor, Brainstem glioma, Brain cancer, Brain tumor (cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal tumors, visual pathway and hypothalamic glioma), Breast cancer, Bronchial adenomas/carcinoids, Burkitt's lymphoma, Carcinoid tumor (childhood, gastrointestinal), Carcinoma of unknown primary, Central nervous system lymphoma, Cerebellar astrocytoma/Malignant glioma, Cervical cancer, Chronic lymphocytic leukemia, Chronic myelogenous

leukemia, Chronic myeloproliferative disorders, Colon Cancer, Cutaneous T-cell lymphoma, Desmoplastic small round cell tumor, Endometrial cancer, Ependymoma, Esophageal cancer, Extracranial germ cell tumor, Extragonadal Germ cell tumor, Extrahepatic bile duct cancer, Eye Cancer (Intraocular melanoma, Retinoblastoma), Gallbladder cancer, Gastric (Stomach) cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal stromal tumor (GIST), Germ cell tumor (extracranial, extragonadal, or ovarian), Gestational trophoblastic tumor, Glioma (glioma of the brain stem, Cerebral Astrocytoma, Visual Pathway and Hypothalamic glioma), Gastric carcinoid, Hairy cell leukemia, Head and neck cancer, Heart cancer, Hepatocellular (liver) cancer, Hodgkin lymphoma, Hypopharyngeal cancer, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi sarcoma, Kidney cancer (renal cell cancer), Laryngeal Cancer, Leukemias ((acute lymphoblastic (also called acute lymphocytic leukemia), acute myeloid (also called acute myelogenous leukemia), chronic lymphocytic (also called chronic lymphocytic leukemia), chronic myelogenous (also called chronic myeloid leukemia), hairy cell leukemia)), Lip and Oral, Cavity Cancer, Liposarcoma, Liver Cancer (Primary), Lung Cancer (Non-Small Cell, Small Cell), Lymphomas, AIDS-related lymphoma, Burkitt lymphoma, cutaneous T-Cell lymphoma, Hodgkin lymphoma, Non-Hodgkin, Medulloblastoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Mouth Cancer, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Chronic Myeloid Leukemia (Acute, Chronic), Myeloma, Nasal cavity and paranasal sinus cancer, Nasopharyngeal carcinoma, Neuroblastoma, Oral Cancer, Oropharyngeal cancer, Osteosarcoma/malignant fibrous histiocytoma of bone, Ovarian cancer, Ovarian epithelial cancer (Surface epithelial-stromal tumor), Ovarian germ cell tumor, Ovarian low malignant potential tumor, Pancreatic cancer, Pancreatic islet cell cancer, Parathyroid cancer, Penile cancer, Pharyngeal cancer, Pheochromocytoma, Pineal astrocytoma, Pineal germinoma, Pineoblastoma and supratentorial primitive neuroectodermal tumors, Pituitary adenoma, Pleuropulmonary blastoma, Prostate cancer, Rectal cancer, Renal cell carcinoma (kidney cancer), Retinoblastoma, Rhabdomyosarcoma, Salivary gland cancer, Sarcoma (Ewing family of tumors sarcoma, Kaposi sarcoma, soft tissue sarcoma, uterine sarcoma), Sézary syndrome, Skin cancer (nonmelanoma, melanoma), Small intestine cancer, Squamous cell, Squamous neck cancer,

Stomach cancer, Supratentorial primitive neuroectodermal tumor, Testicular cancer, Throat cancer, Thymoma and Thymic carcinoma, Thyroid cancer, Transitional cell cancer of the renal pelvis and ureter, Urethral cancer, Uterine cancer, Uterine sarcoma, Vaginal cancer, Vulvar cancer, Waldenström macroglobulinemia, and/or Wilm's tumor.

The small molecule-EVs as per the present invention may be administered to a human or animal subject via various different administration routes, for instance auricular (otic), buccal, conjunctival, cutaneous, dental, electro-osmosis, endocervical, endosinusial, endotracheal, enteral, epidural, extra-amniotic, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-arterial, intra-articular, intrabiliary, intrabronchial, intrabursal, intracardiac, intracartilaginous, intracaudal, intracavernous, intracavitary, intracerebral, intracisternal, intracorneal, intracoronal (dental), intracoronary, intracorporus cavernosum, intradermal, intradiscal, intraductal, intraduodenal, intradural, intraepidermal, intraesophageal, intragastric, intragingival, intraileal, intralesional, intraluminal, intralymphatic, intramedullary, intrameningeal, intramuscular, intraocular, intraovarian, intrapericardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratendinous, intratesticular, intrathecal, intrathoracic, intratubular, intratumor, intratym panic, intrauterine, intravascular, intravenous, intravenous bolus, intravenous drip, intraventricular, intravesical, intravitreal, iontophoresis, irrigation, laryngeal, nasal, nasogastric, occlusive dressing technique, ophthalmic, oral, oropharyngeal, other, parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (inhalation), retrobulbar, soft tissue, subarachnoid, subconjunctival, subcutaneous, sublingual, submucosal, topical, transdermal, transmucosal, transplacental, transtracheal, transtympanic, ureteral, urethral, and/or vaginal administration, and/or any combination of the above administration routes, which typically depends on the disease to be treated and/or the characteristics of the small molecule drug or the EV population as such.

The methods of loading small molecules into EVs described herein are highly efficient and easily scalable, and allow for the rapid production of small molecule-loaded EVs in quantities needed for therapeutic administration. In certain

embodiments of the foregoing aspects, loading of the EVs with the small molecule occurs in 30 minutes or less, e.g. 5 minutes or less. In some embodiments, loading of the EVs occurs in 30 minutes, 20 minutes, 15 minutes, 10 minutes, 5 minutes, or 1 minute. In certain embodiments, at least 80% of the EVs incubated with a small molecule CPP conjugate or CPP complex are loaded with the small molecule. In a preferred embodiment, at least 90% of the EVs incubated with a small molecule CPP complex/conjugate are loaded with the small molecule. In exemplary embodiments, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or more of the EVs incubated with the small molecule conjugates/complexes are loaded with the small molecule. In one embodiment at least 99% of the EVs incubated with the small molecule-CPP complex/conjugate are loaded with the small molecule.

The methods of the present invention may also comprise exposing the EV source cells to serum starvation, hypoxia, bafilomycin, or cytokines such as TNF-alpha and/or IFN-gamma, in order to influence the yield or properties of the resulting EVs into which small molecules are subsequently loaded. The EV production scale and timeline will be heavily dependent on the EV-producing cell or cell line and may thus be adapted accordingly by a person skilled in the art.

The methods for loading small molecule drugs into EVs may further comprise a purification step, wherein the EVs are purified through a procedure selected from the group of techniques comprising liquid chromatography (LC), high-performance liquid chromatography (HPLC), spin filtration, tangential flow filtration, hollow fiber filtration, centrifugation, immunoprecipitation, flow field fractionation, dialysis, microfluidic-based separation, etc., or any combination thereof. In an advantageous embodiment, the purification of the EVs is carried out using a sequential combination of filtration (preferably ultrafiltration (UF), tangential flow filtration or hollow fiber filtration) and size exclusion liquid chromatography (LC). This combination of purification steps results in optimized purification, which in turn leads to superior therapeutic activity. Further, as compared to ultracentrifugation (UC), which is routinely employed for purifying EVs such as exosomes, sequential filtration-chromatography is considerably faster and possible to scale to higher manufacturing volumes, which is a significant drawback of the current UC methodology that dominates the prior art. Another

advantageous purification methodology is tangential flow filtration (TFF), which offers scalability and purity, and may be combined with others types of purification techniques such as filtration. Purification techniques are typically deployed prior to loading exposing the EVs to the small molecule-CPP conjugates and/or complex, in order to avoid interference from e.g. serum proteins, or unspecific loading into EVs having undesirable characteristics or features. A typical workflow for the production of small molecule-carrying EVs is (1) creation of a stable cell line expressing EVs having a targeting moiety displayed on their surface, (2) in an optional step purification of large quantities of such genetically engineered EVs, (3) introduction of at least one small molecule drug into the EVs in question, with the aid of at least one CPP. CPPs may be mixed with the small molecule drug in a separate vessel and/or e.g. in a microfluidic device prior to exposure to EVs, for instance to enable complex formation prior to interaction with the EV population. Alternatively, complex formation or covalently conjugated CPP-small molecules may be mixed directly with the EVs, typically in the form or purified EV populations.

It shall be understood that the above described exemplifying aspects, embodiments, alternatives, and variants can be modified without departing from the scope of the invention. The invention will now be further exemplified with the enclosed examples, which naturally also can be modified considerably without departing from the scope and the gist of the invention.

Examples

Example 1: Immune cell-derived EVs loaded with CPP-doxurubicin conjugates

Peripheral blood mononuclear cells (PBMCs) are extracted from whole blood and plated at an appropriate density in cell media. The cell media is removed after 24 hours and the plate is washed with PBS 3 times. New fresh EV-depleted media or serum free media is added. EVs are purified from the conditioned media.

The media that the cells are grown in is normally depleted of foreign EVs and microparticles by ultracentrifugation at 110 000 g overnight before incubation with the cells. Alternatively, serum free media is applied in its place, such as OptiMEM or DMEM.

Conditioned media from the PBMC culture is purified using different techniques, in this case ultrafiltration with sequential LC, or tangential flow filtration (TFF).

The CPP penetratin was covalently conjugated to the cardiotoxic anticancer agent doxorubicin as previously described by Shi *et al.*, Int J Nanomedicine, 2012; 7:1613-21.

A composition comprising a suitable concentration (for instance, 10^{12} (i.e. 10 trillion) particles/ml) of PBMC EVs obtained from the TFF or UF/LC purification steps were mixed with a buffer containing penetratin-doxorubicin conjugates at a concentration of 1 mM. After a 30-minute incubation, non-loaded penetratin-doxorubicin conjugates are removed from the loaded EVs using a simple filtration step using a filter having a pore size of 10 kDa.

The cytotoxicity of free doxorubicin, EVs loaded with penetratin-doxorubicin conjugates, and free penetrating-doxorubicin conjugates were determined using the MTT assay. MDA-MB-231 or MCF7 cells (1×10^5 cells/100 µl/well) were cultured in 96-well plates at 37 °C and 5% CO_2. Aqueous drug solutions as per above were dissolved in culture medium at final concentrations of 0.1, 0.5, 1, 5, 10 and 20 µM. After an incubation time of 4 h, the MTT solution (2 mg/ml PBS) was added, the plates were incubated for 4 h, and the cells were lysed with 50% N,N-dimethylformamide containing 20% SDS, pH 4.5. The absorbance at 570 nm was measured for each well by the SpectraMax M5 instrument (Molecular Devices, CA). The absorbance of control cells was taken as 100% viability, and the values of the treated cells were calculated as a percentage of control. The results are shown in Figure 1, indicating that EVs loaded with penetratin-doxorubicin conjugates display similar anticancer effects as free CPP-doxorubicin conjugates, warranting further *in vivo* investigation.

Example 2: In vivo evaluation of penetratin-doxorubicin conjugates loaded into immune cell EVs

The penetratin-doxorubicin EVs in Example 1 were tested in an *in vivo* tumor model, using female DBA/2 mice (weighing 16–20 *g*). On day zero, five groups of mice were inoculated via i.p. injection with L1210 tumour cells (2.5×10^6) in 0.5 ml of

RPMI1640. Treatment was initiated 1 day after injection of tumour cells and was administrated as a single i.v. dose via the lateral tail vein. The animals were treated with penetratin-doxorubicin conjugates, EVs comprising penetratin-doxorubicin conjugates, empty EVs, and free doxorubicin. Survival time was recorded in days after tumour injection. The mean and median survival time and the statistical significance of the results were determined employing a two-tailed Wilcoxon's ranking test. All data obtained for repeated experiments were pooled and utilised for statistical analysis. Results are shown in Figure 2, indicating that EVs loaded with penetratin-doxorubicin conjugates exhibit similar efficacy as free penetratin-doxorubicin conjugates. However, the cardiotoxicity of doxorubicin was reduced considerably upon EV-mediated delivery (data not shown).

Example 3: In vivo evaluation of DC-EVs loaded with CADY1-paclitaxel complexes

EVs are obtained as in Example 1 but from dendritic cells (DCs). Paclitaxel and the amphipathic CPP CADY-1 (GLWWKAWWKAWWKSLWWRKRKRKA) were mixed at various molar ratios and incubated at 4 °C for 1 hour. Varying amounts of CADY-1 were used (100 µg/ml, 500 µg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml), whilst the concentration of paclitaxel remained at 100 µg/ml. The final molar ratios of paclitaxel to CADY-1 were 1:1, 1:5, 1:10 and 1:20, respectively. The same mouse model as in Example 2 was utilized, but this time with EVs which had been incubated with paclitaxel-CADY1 complexes were used, after the free paclitaxel-CADY1 complexes had been removed through filtration. EVs comprising paclitaxel-CADY1 complexes, paclitaxel-CADY1 complexes alone, empty EVs and free paclitaxel were tested *in vivo* in the tumor model as above. In analogy with the results seen with the penetratin-doxorubicin EVs, EVs comprising paclitaxel-CADY1 complexes exhibited strong antitumor effects (Figure 3).

Example 4: In vivo evaluation of MSC-EVs loaded with stearylated TP10-azathioprine complexes for the treatment of colitis

EVs were produced from a culture of mesenchymal stromal cells of bone marrow and Wharton's jelly origin. The purine analogue azathioprine was mixed at various molar

ratios with the cell-penetrating peptide stearyl-TP10 and incubated at 4°C for 10 minutes, 30 minutes, 2 hours, and 6 hours. Varying amounts of the CPP were used, whilst the concentration of azathioprine remained constant. The complexes obtained at the optimal molar ratio of CPP to small molecule drug were subsequently mixed with purified populations of MSC-EVs and allowed to incubate at 37°C for 10 minutes, 30 minutes, 2 hours, and 6 hours. Maximum loading of EVs was obtained within 30 minutes and azathioprine-loaded MSC-EVs were thus subsequently administered to mice with TNBS-induced colitis.

TNBS-induced colitis simulating the cytokine storm, the diarrhoea, weight decrease, and gut inflammation seen in IBD patients. 24 mice were divided into four treatment groups, with 6 mice per group. The mice were pre-sensitized by applying 150 µl of a olive oil-acetate solution with 2% TNBS, on the skin, 1 week prior to colitis induction. Colitis was then induced by giving a rectal infusion of 100 µl solution containing 1.5 % TNBS in 40 % ethanol. Immediately post colitis induction, 30 µg azathioprine-containing EVs in 200 µl were administrated intravenously in the tail vein and the bodyweight was recorded daily. Mice treated with azathioprine-TP10 EVs displayed the quickest recovery after colitis reduction, as can be seen in Figure 4.

Claims

1. A method for loading an extracellular vesicle (EV) with a small molecule agent, comprising exposing a population of EVs to at least one small molecule agent and at least one cell-penetrating peptide (CPP).
2. The method according to claim 1, wherein the at least one small molecule agent and the at least one CPP are present in the form of covalent conjugates, non-covalent complexes or a combination thereof.
3. The method according to claim 2, wherein the covalent conjugate between a CPP and a small molecule agent comprises a bond selected from the group comprising an ester bond, an amide bond, a disulfide bond, a thioether bond, a biotin-streptavidin linkage, or any combination thereof.
4. The method according to claim 2, wherein the non-covalent complexes between CPPs and small molecule drugs are present in the form of nanoparticles with positive or negative zeta potential.
5. The method according to any one of the preceding claims, wherein the loading of the complexes and/or conjugates is enhanced by the inclusion of a transfection reagent such as a liposome and/or a lipid nanoparticle.
6. A method for loading an EV with a small molecule agent, comprising the steps of:
    a. exposing a population of EV source cells to at least one small molecule agent and at least one cell-penetrating peptide (CPP); and,
    b. harvesting EVs produced by the EV source cells, wherein the EVs comprise said small molecule agent.
7. An EV obtainable by the methods of any one of the preceding claims.
8. An EV comprising at least one small molecule agent conjugated to or complexed with at least one CPP.
9. An EV comprising at least one small molecule agent, wherein the at least one small molecule agent is released from at least one CPP conjugate and/or at least one CPP complex inside the EV.
10. The EV according to any one of claims 7 to 9, wherein the small molecule drug is an anticancer agent, a cytostatic agent, a DNA or RNA intercalator, a splicing modulator, a tyrosine kinase inhibitor, a statin, an NSAID, an

antibiotic, an antifungal agent, an antibacterial agent, an anti-inflammatory agent, an anti-fibrotic, an antihypertensive, an aromatase inhibitor, an esterase inhibitor, an anticholinergic, an SSRI, a BKT inhibitor, a PPAR agonist, a HER inhibitor, an AKT inhibitor, a BCR-ABL inhibitor, a signal transduction inhibitor, an angiogenesis inhibitor, a synthase inhibitor, an ALK inhibitor, a BRAF inhibitor, a MEK inhibitor, a PI3K inhibitor, a neprilysin inhibitor, a beta2-agonist, a CRTH2 antagonist, an FXR agonist, a BACE inhibitor, a sphingosine-1-phosphate receptor modulator, a MAPK inhibitor, an Hedgehog signaling inhibitor, an MDM2 antagonist, an LSD1 inhibitor, a lactamase inhibitor, a TLR agonist, a TLR antagonist, an IDO inhibitor, an ERK inhibitor, a Chk1 inhibitor, and any combination thereof.

11. The EV according to any one of claims 7 to 10, wherein the CPP is selected from the group comprising transportan, transportan 10, penetratin, CADY peptides, MTS, VP22, MAP, KALA, PpTG20, proline-rich peptides, MPG peptides, PepFect peptides, Pep-1, L-oligomers, calcitonin-peptides, arginine-rich CPPs such as poly-Arg, Tat, and any combination thereof.
12. The EV according to any one of claims 7 to 11, further comprising a targeting moiety.
13. The EV according to claim 12, wherein the targeting moiety comprises a sequence of amino acids expressed as a fusion protein with an EV polypeptide.
14. A method of delivering a small molecule drug comprising exposing a target cell to an EV according to any one of claims 7 to 13.
15. A method of altering the pharmacokinetic or pharmacodynamics profile of a small molecule drug, comprising loading the small molecule into an EV according to the methods of any one of claims 1 to 6.
16. A pharmaceutical composition comprising EVs according to any one of claims 7 to 13 and a pharmaceutically acceptable excipient.

Intellectual Property Office

Application No:	GB1611988.5	**Examiner:**	Dr Jeremy Kaye
Claims searched:	1-16	**Date of search:**	24 March 2017

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-5, 7-11, 14-16	Biomaterials, Vol.35, 2014, Yang Yanfang, et al., "PEGylated liposomes with NGR ligand...", pp.4368-4381
X	1-5, 7-11, 14-16	Drug Delivery, Vol.23, 2016, Lin Wen, et al., "Thermosensitive magnetic liposomes...", pp.3436-3443 Available online: http://www.tandfonline.com/doi/full/10.1080/10717544.2016.1189983; epub 07/06/2016
X	6, 12 & 13	WO2016/044947 A1 (EXERKINE CORP.) see esp. para.[0083]

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

The following online and other databases have been used in the preparation of this search report

WPI, EPODOC, TXTA, BIOSIS, MEDLINE

International Classification:

Subclass	Subgroup	Valid From
None		