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(54) Title: LIPOSOMAL SYSTEM WITH KILLER TNF-APOPTOSIS INDUCED LIGAND (KILLERTRAIL), PRO-APOPTOTIC-DIRECTING

(57) Abstract: The present invention relates to the development of transport systems of the liposomal type which have PEGylated compounds conjugated with derivatives of the family of Tumor Necrosis Factor cytokines on the surface of the vesicular structure. The conjugation strategy is to bind the protein, preferably TRAIL, more preferably KillerTRAIL, in monomeric form, to the polyethylene glycol (PEG) via a thiol group (-SH) of the polypeptide chain.

Liposomal system with Killer TNF-apoptosis induced ligand (KillerTRAIL), pro-apoptotic-directing

Technical field of the invention

The present invention has as its object the development of transport systems of the liposomal type which have on the surface of the structure vesicular PEGylated compounds conjugated with derivatives of the TNF (family of cytokines *Tumor Necrosis Factor)*. In particular, theinvention relates to the use in therapy and diagnosis of the conjugate of a protein of the TRAIL family and related mutants for the diagnosis and treatment of various tumors in mammals and humans.

To date, at least eight members of this same family are known: LTα, KillerTRAILβ, LTβ, the CD20 ligands; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), in addition to KillerTRAIL, TNF-apoptosis induced ligand (KillerTRAIL).

Among all the various members of the family, KillerTRAIL was considered suitable for the mode of stabilization, delivery and direction by means of polymer conjugation on the surface of a liposomal nanovector.

With the aim of overcoming the critical problems typical of therapeutic proteins, such as rapid elimination, toxic degradation and accumulation (formation of immune complexes), site-specific direction and co-administration of synergistic active ingredients, KillerTRAIL has been derivatized in a single process with a polymer chain on the surface of liposomal type nanovectors. The derivatization exemplified below can be applied similarly to the other members of the TRAIL family such as TRAILR1 (DR4; Apo2; CD261; 5 TNFRSF10A) [1], TRAIL-R2 (DR5, KILLER, TRICK2A, 10 TRICK2B, CD262, TNFRSF10B), TRAIL-R3 (DcR1, LIT, TRID, CD263, TNFRSF10C), TRAIL-R4 (DcR2; TRUNDD; CD264; TNFRSF10D), and osteoprotegerin (OPG; OCIF; TNFRSF11B), LZ-TRAIL, IZ-TRAIL, FLAG / His- TRAIL, TRAIL-R1 (Mapatumumab),

TRAIL-R2 (Lexatumumab; HGS-TR2J; Apomab, AMG655, LBY135; CS-1008 or human TRA-8), TNFSF10, TL2; APO2L; CD253; Apo-2L, LTα, KillerTRAILβ, LTβ, ligands of CD20; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), TNFSF10, TL2; APO2L; CD253; Apo-2.

The first stage of processing consists in the purification of the KillerTRAIL protein by dialysis (regenerated cellulose cut-off 10,000 Da) to eliminate the solution in which the protein has been stored (salts, ethylenediaminetetraacetic acid EDTA, dithiothreitol DTT) in which it is marketed. Subsequently, a treatment of the protein solution with only 8 mM DTT is carried out at room temperature for 1 hour in order to avoid the spontaneous formation of disulfide bridges that would lead to the formation of the protein in the form of a trimer. To maintain optimal conditions for conjugate production, it further purifies the protein solution from the DTT by desalting chromatography column. Under these experimental conditions the KillerTRAIL protein is found in monomeric form. At this point the KillerTRAIL protein in monometric form is conjugated by the thiol group of the cysteine in position 230 of the peptide chain of the KillerTRAIL with an alkoxy-PEG through the maleimide reactive group of the PEG. The reaction can take place in an aqueous environment in a single step, preferably after the prior purification of the native KillerTRAIL, for example by dialysis against PBS (pH 7.4) containing EDTA to allow the elimination of the dithiothreitol (DTT) used as a preservative in the preservation of KillerTRAIL. and in the case of the reaction, to avoid the spontaneous formation of disulfide bridges and aggregation between the protein monomers.

To make the compounds of the invention, hetero-bifunctional polyethylene glycol (PEG) is used, conjugated at one end with a phospholipid, such as DSPE (distearoyl-phosphatidyl-ethanolamine), and activated at the other end with a maleimide group (MAL). Conjugation is carried out on the surface of the liposomal vector, the formulation and preparation of which is described below.

One of the strategies proposed for the delivery of the protein belonging to the family of cytokines TNF (*Tumor Necrosis Factor*), preferably TRAIL, more preferably KillerTRAIL, is the binding of the conjugate on the surface of vesicular-type structures to form liposomal systems characterized by *targeting* specific, where by *targeting* specificwe mean the ability of the aforementioned liposomes to selectively bind the receptors of the TRAIL family, more specifically KillerTRAIL, present on the surface of cells and tissues, and in particular TRAIL-R1 (DR4; Apo2; CD261; TNFRSF10A) [1], TRAIL-R2 (DR5, KILLER, TRICK2A, 10 TRICK2B, CD262, TNFRSF10B) [1-3], TRAIL-R3 (DcR1, LIT, TRID, CD263, TNFRSF10C) [1,2,4], TRAIL -R4 (DcR2; TRUNDD; CD264; TNFRSF10D) [5], and osteoprotegerin (OPG; OCIF; TNFRSF11B) [6-7].

This conjugation does not preclude its activity, on the contrary, it reduces its short and long-term toxicity by remaining within the therapeutic window, increasing the half-life of the protein itself and extending the time between one administration and another. The presence of the targeted protein with therapeutic activity on the surface of the liposomal system allows a specific targeting on tumor cells that over-express the death receptor, an enhanced therapeutic activity, site specific, of activation of the cascades of cell death by apoptosis, and last but not least the possibility of co-vehiculating within the liposomal system one or more active ingredients with antitumor activity and synergistic action, which guarantees the efficacy of the therapy even on TRAIL-resistant cells.

The conjugation strategy object of the present invention is that of binding the protein, TRAIL and related mutants, more preferably KillerTRAIL, to polyethylene glycol (PEG). The PEG chain used is an integral part of the vesicular structure of the liposomal system thanks to the presence in the *lipid* bilayer of a PEGylated-phospholipid. While the phospholipid, due to its structure and its chemical-physical characteristics, will be inserted in the *lipid* bilayer, the PEG chain will be exposed in whole or in part on the

surface of the liposomal vesicle towards the outside, and the exposed end will contain the group maleimide (MAL) reactive.

The system thus produced has been characterized both for its chemical-physical and biological properties.

The conjugation of the protein belonging to the TNF (family of cytokines *Tumor Necrosis Factor*), preferably TRAIL, more preferably KillerTRAIL, occurs through a thiol group (-SH) present on the polymer chain of the protein.

The PEG can be an alkoxy-polyethylene glycol, preferably methoxy-polyethylene glycol (mPEG) derivatized at one end with a phospholipid, preferably distearoyl-phosphatidyl-ethanolamine (DSPE) and at the other end with a reactive group towards the thiols. This approach allows to obtain an amphiphilic and stable derivative, which is able to overcome the stability problems, the aggregation phenomena and the low half-life linked to the native protein, thus obtaining an improvement of the biopharmaceutical and pharmacokinetic parameters and a better antitumor activity and selectivity compared to the native protein.

Furthermore, the amphipathic characteristics of the compound, object of the invention, have also made it possible to create liposomal systems, which present the native protein exposed on the surface, capable of improving the stability, biodistribution, activity of the protein, while maintaining its therapeutic and targeting effect.

This system guarantees the possibility of co-vehiculating hydrophilic, lipophilic or amphipathic drugs within the nanosystem that can have a synergistic action and reduce the phenomena of resistance to anticancer therapy. In fact, one of the problems encountered in the clinical development of this protein is the resistance to pro-apoptotic therapy. This phenomenon is present on tumor tissues that express on their surface the receptor of the TNF-family of cytokines *apoptosis induced ligand* (KillerTRAIL). These

defense mechanisms, still poorly understood, inhibit the pro-apoptotic pharmacological action of compounds of the TRAIL family of cytokines as reported in the literature [8,9].

To realize the nanosystems of the invention, hetero-bifunctional polyethylene glycol (PEG) is therefore used, conjugated at one end with a phospholipid such as, for example, DSPE (distearoyl-phosphatidyl-ethanolamine) and activated at the other end to be reactive against the thiol groups present on the protein. Preferably the group is the maleimide group (MAL).

The use of this derivative promotes the synthesis of an amphipathic compound, consisting of phospholipid-PEG-protein, which is inserted in the vesicular structure of the liposomes and in particular in the *bilayer* phospholipidand presents the therapeutic protein on the surface of the liposomes.

The presence of the protein on the surface of liposomes allows to obtain the targeting and site-specific action towards tumor cells and tissues that express on their surface the receptors of the TRAIL family, more specifically the KillerTRAIL present on the surface of cells and tissues, and in particular TRAIL-R1 (DR4; Apo2; CD261; TNFRSF10A) [1], TRAIL-R2 (DR5, KILLER, TRICK2A, 10 TRICK2B, CD262, TNFRSF10B) [1-3], TRAIL-R3 (DcR1, LIT, TRID, CD263, TNFRSF10C) [1,2,4], TRAIL-R4 (DcR2; TRUNDD; CD264; TNFRSF10D) [5], and osteoprotegerin (OPG; OCIF; TNFRSF11B) [6,7]. LZ-TRAIL, IZ-TRAIL, FLAG / His-TRAIL, TRAIL-R1 (Mapatumumab), TRAIL-R2 (Lexatumumab; HGS-TR2J; Apomab, AMG655, LBY135; CS-1008 or 25 human TRA-8), TNFSF10, TL2; APO2L; CD253; Apo-2L, LTα, KillerTRAILβ, LTβ, ligands of CD20; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), TNFSF10, TL2; APO2L; CD253; Apo-2.

BACKGROUND ART

In recent years, pro-apoptotic proteins have met with great success, largely due to their powerful antitumor activity *in vitro* and *in vivo*. The most promising proteins were

those of the TRAIL family. TRAIL (also known as TNFSF10, TL2; APO2L; CD253; Apo-2L) is a member of the TNF ligand family.

Its most promising mutant in therapy proved to be the protein known and marketed as KillerTRAIL. The human KillerTRAIL is a known compound and is present on the market (http://www.enzolifesciences.com/ALX-201-073/killertrail-protein-soluble-human-recombinant/) .The sequence of the monomeric human KillerTRAIL was solved by the inventors of the present invention by means of tandem mass spectrometry (MS / MS) and a mass mapping method based on the strategy of the extracted ion and is shown below: specific sequence of the TRAIL (Seq ID No 1: **TSEE**TISTVQ EKQQNISPLV RERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG) conjugated through the head residues Seq ID No 4: **TSEE** to the specific sequence of the peptide Killer (Seq ID No 2: HHHHHHYLMYGTVTLGY).

Unfortunately, no clinical studies have continued in this area due to the problems encountered during the administration of KillerTRAIL. The rapid clearance, the high toxicity, most likely due to the protein trimerization after systemic administration, the formation of immune complexes and the therapeutic resistance by tumor tissues, make KillerTRAIL a little used drug in the clinical field.

To optimize the clinical use of this protein, mixed polymer-lipid nanoaggregates containing KillerTRAIL have been synthesized, capable of complexing lipophilic and amphipathic drugs and directing the pro-apoptotic protein KillerTRAIL, which has therapeutic and targeting properties, towards cells that over-express specific receptors for the native protein on their surface. The use of these nanoaggregates is based on the principle that macromolecules, with amphiphilic characteristics, can self-aggregate and

form micellar structures with the lipophilic portion forming a core towards the inside of the aggregate (lipid component) and the hydrophilic portion towards the external aqueous compartment (directing protein).

The need to solve the above problems, personalize the therapy, increase its therapeutic efficacy and reduce its toxicity has, however, remained unchanged.

KillerTRAIL has been studied as a potential anticancer compound for several years. Effective anticancer therapy aims to develop highly selective drugs with high therapeutic efficiency and reduced side effects [10].

The KillerTRAIL protein is able to induce apoptosis in different types of cancer cells resistant to conventional chemotherapy and radiotherapy, without causing significant toxic effects on healthy cells of patients [11,12]. However, primary tumor cells rapidly develop resistance to chemotherapy treatment with KillerTRAIL, despite the expression on their surface of pro-apoptotic receptors, specific for this protein, making this drug ineffective in cancer therapy.

This resistance is likely caused by multiple mechanisms, and includes overexpression of Bcl-2 family proteins, especially XIAP, overexpression of protein kinase B (PKB / Akt), deletion of the Bax gene, and caspase mutations., as well as many other resistance factors [13-16]. Despite all these hypotheses, the mechanisms underlying the resistance of primary tumor cells to KillerTRAIL are still the subject of numerous experimental investigations, and numerous researches have aimed to develop chemotherapy protocols in which KillerTRAIL has been used in combination with other anticancer drugs to enhance the therapeutic activity of this compound [17,18].

The TNF-apoptosis-inducing ligand (TRAIL, Apo2L, CD253, TNFSF10) is a type of trans-membrane protein II of about 34 kDa, discovered by two different groups in the years 1995/1996 [19,20]. TRAIL is a known protein (https://www.uniprot.org/uniprot/P50591) which consists of an amino acid sequence of

281 aa in the human form and 291 aa in the mouse form, which share a degree of homology of 65%, and it has a molecular weight of about 32.5 kDa. TRAIL is characterized by a transmembrane domain, a cysteine-rich extracellular C-terminal domain conserved among members of the same family, and a cytoplasmic N-terminal domain that instead presents unique characteristics.

The members of the TRAIL family, to which KillerTRAIL also belongs, such as KillerTRAIL-α, FasL, TRAIL, which are also called cell death ligands, are able to activate a cell death program following binding with specific membrane receptors. TRAIL, for example, is a homotrimeric ligand able to interact with specific members of the KillerTRAIL receptor family thanks to the presence of extracellular CRD domains [21].

TRAIL and KillerTRAIL differ for a polyhistidine tag (HisTag), present on the KillerTRAIL peptide chain which increases its therapeutic function as well as for the specific peptide sequence of the Killer protein.

Tandem mass spectrometry (MS / MS) experiments and a mass mapping method based on the extracted ion strategy performed by the inventors of the present invention on the commercial product Enzo Life allowed to solve the specific sequence of KillerTRAIL with sequence coverage of 90 %. In particular, the two experimental methods used have shown that the KillerTRAIL commercial product has within its structure the specific sequence of TRAIL(SeqID No 1: **TSEE**TISTVQ EKQQNISPLV RERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG), which is the protein sequence mapped by MALDI analysis, with a sequence coverage of 97.8%, and the N-terminal stretch of the protein (Seq ID No 4: TSEE), highlighted in bold, indicates the binding sequence with the Killer peptide, and the specific sequence of the Killer peptide is Seq ID No 2: HHHHHHYLMYGTVTLGY,

consisting of a 6 histidine tag, thepeptide *linker* and the first four amino acids of the TRAIL protein (in bold).

Like most members of the TNF cytokine superfamily, KillerTRAIL can be cut from the cell surface by metalloproteases to form a soluble molecule [22]. TRAIL, the other members of the TRAIL family [23] and KillerTRAIL, in their active form, form trimeric macromolecules and selectively bind to five distinct receptors: TRAIL-R1 (DR4; Apo2; CD261; TNFRSF10A) [1], TRAIL-R2 (DR5, KILLER, TRICK2A, TRICK2B, CD262, TNFRSF10B) [18-20], TRAIL-R3 (DcR1, LIT, TRID, CD263, TNFRSF10C) [1,2,4], TRAIL-R4 (DcR2; TRUNDD; CD264; TNFRSF10D) [5], and osteoprotegerin (OPG; OCIF; TNFRSF11B) [6,7].

TRAIL and KillerTRAIL are active against various pathologies in which cells and tissues express on their surface the receptors of the TNF cytokine superfamily. Indeed, it has been reported in the literature that Apo2L / TRAIL, and therefore also KillerTRAIL, can play a role in the modulation of the immune system, including autoimmune diseases such as arthritis [24-28]. Furthermore, it has also been reported that soluble forms of Apo2L / TRAIL, and therefore also KillerTRAIL, induce apoptosis *in vitro* in different types of cancer cells, including cancers of the colon, lung, breast, prostate, kidney, ovaries. and central nervous system, as well as melanoma, leukemia and multiple myeloma [29-38].

The targeting and therapeutic potentials of this ligand are manifold. However, the problem of its toxicity remains, linked to the aggregation of the protein after systemic administration, a phenomenon similar to that which occurs for other therapeutic proteins [39], which often prevents the continuation of therapy due to the triggering of an immune response towards it. cure [39-41].

In addition to recombinant human proteins with a structure similar to KillerTRAIL, which show potent antitumor activity and selectivity against tumor cells that express on

their surface the TRAIL-Rs receptor system [42], antibodies against the KillerTRAIL receptor, which function as agonists, are being tested in various experimental studies for their potential therapeutic action. The use of selective TRAIL-R1- or TRAIL-R2- variants could allow for better tumor-specific therapy by blocking the effect mediated by the receptor antagonist, resulting in greater efficacy with fewer side effects thanTRAIL *wild type* [43 -47].

Recombinant versions of TRAIL, such as the one called KillerTRAIL, which has a dipole-histidine tag (HisTag), or reduced structures based on leucine and isoleucine, as well as the non-tagged versions, have been produced and tested to evaluate their efficacy and selectivity in different tumor models. While the non-Tag versions appeared to have high selectivity towards tumor cells, but less efficacy, the KillerTRAIL-tagged versions showed greater efficacy for the formation of complex structures with higher affinity. At the same time, these KillerTRAIL derivatives showed *in vitro* slightly higher toxicity towards normal cells, such as human hepatocytes[48,49].

LZ-TRAIL and IZ-TRAIL represent an intermediate state and have similar efficacy to FLAG / His-TRAIL *in vitro*, but do not have the same toxicity as previously reported compounds [50-52]. Initially, it was hypothesized that the KillerTRAIL aggregates could be hepatotoxic [53]. However, some research has reported conflicting results for the toxic effect of KillerTRAIL on the liver. These experimental results are currently the subject of numerous studies. However, it is not clear whether thehepatotoxicity *in vitro* of the different KillerTRAIL variants can also occur *in vivo* [54].

The use of monoclonal antibodies with agonistic activity, as therapeutic agents, represents one of the emerging strategies in anticancer therapy. Monoclonal antibodies selective for the TRAIL-R1 receptor (Mapatumumab) or TRAIL-R2 (Lexatumumab; HGS-TR2J; Apomab, AMG655, LBY135; CS-1008 or humanized version of TRA-8) are currently undergoing clinical trials [50, 55.56].

However, the need to solve the problems described so far, to personalize the therapy, to increase its therapeutic efficacy and reduce its toxicity, has remained unresolved to date.

If not specifically excluded in the detailed description that follows, what is described in this chapter is to be considered as an integral part of the detailed description of the invention.

Summary of the invention

The inventors have for this reason developed and experimentally verified new strategies for the delivery of compounds that belong to the members of the TRAIL family, to which the KillerTRAIL also belongs, such as KillerTRAIL, KillerTRAIL-α, FasL, TRAIL, in order to to obtain a modulation of the biopharmaceutical characteristics of the family of these proteins (such as TRAIL, KillerTRAIL) without however modifying their binding capacity with the cell substrate.

The inventors have in fact found that a liposomal vector-PEG-protein conjugation, in which the protein is TRAIL or its mutant, in particular KillerTRAIL, unexpectedly does not affect the binding properties of the native protein with the receptor site.

For this purpose, the inventors have also solved the specific sequence of human KillerTRAIL by means of tandem mass spectrometry (MS / MS) and a mass mapping method based on the strategy of the extracted ion.

Therefore, the object of the present invention is the realization of a new therapeutic formulation, stable, biocompatible and biodegradable whose vesicular structures, formed by liposomes covered on their surface by PEG chains to which the therapeutic protein belonging to the TRAIL family is linked, in particular KillerTRAIL, are non-toxic, easy to reproduce on an industrial scale (*scale-up*) and versatile from a therapeutic point of view.

The patent document WO2004001009 describes variants of the Apo2L / TRAIL protein and reports the derivatization with polyethylene glycol (PEG) performed on cysteines inserted in the chain of the variants of TRAIL in position 170 or 179 (indicated respectively in the document as PEG-R170C-Apo 2L and PEG -K179C-Apo 2L). PEGylation, which affects the cysteine residues, is considered useful only if carried out on the cysteines inserted in the variants in position 170 and 179 in order to preserve the cysteine in position 230, considered fundamental for biological activity and therefore not modifiable in order not to modify the homotrimeric structure and the corresponding activity of the protein.

This binding site has been shown to be important for protein activity. In fact, the substitution of cysteine 230 (Cys 230) determines an 8-fold reduction in apoptotic activity. In addition, removal of the coordinated metal from the homotrimer cystines causes a 7-fold decrease in affinity for DR5 and a 90-fold reduction in apoptotic activity. Furthermore, after the removal of the coordination Zn, the cysteines can be oxidized and Apo-2L dimers bonded with disulfide bridges are formed, which reduce apoptotic activity. The binding site of the coordination metal is hidden within the structure of the Apo-2L trimer and thus prevents its interaction with the receptor.

Furthermore, as reported in WO2004001009 and in the literature prior to it (Hymowitz SG et al., Biochemistry, 2000; 39 (4): 633-40; Bodmer et al., J. Biol. Chem., 2000; 275 (27): 20632-7), the bond with the divalent metal ion appears to be of fundamental importance to maintain the structure of the trimer and the stability of Apo-2L and the consequent biological activity. In document WO2004001009, therefore, a derivatization with PEG is carried out on the cysteines inserted in the polypeptide variants of Apo2L in order to maintain the trimerization and evaluate their therapeutic efficacy. The derivatizations with PEG considered particularly interesting are those

carried out on the mutations in which the cysteine residues are in position C170 and C179.

The insertion of the further cysteines in addition to the naturally present Cys 230, and their corresponding derivatization through PEG in effect produce an improvement in the therapeutic activity, which however does not seem to be considered by the inventors as optimal for use in the pharmaceutical field. In fact, despite some potentially encouraging results, albeit obtained by modifying the peptide chain of the native protein, no other research published later has been carried out. In fact, making changes on the native protein chain by substituting one or more amino acids involves the aboriginal manipulation of the genetic material. This situation complicates processing and significantly increases production costs and administration risks (in fact, the metabolic characteristics and potential adverse effects, especially hepatic, of the varied protein are unknown). Furthermore, the improvement of the activity obtained through the changes reported in the aforementioned document is not considered such as to justify the risk of use.

To improve the biopharmaceutical properties, increase the therapeutic activity of the TRAIL family of pro-apoptotic proteins and related mutants, in particular KillerTRAIL, and reduce the resistance of primary tumor cells, the pro-apoptotic protein was reduced from the trimeric to the monometric form. and modified by conjugation with a phospholipid-PEG derivative, preferably distearoyl-phosphatidyl-ethanolamine-polyethylene glycol (DSPE-PEG), through a *linker*, such as a maleimide(MAL) *linker.* The synthesis process can be performed with simple reaction steps both on the preformed liposomal vesicles and during their preparation. In both cases the therapeutic protein of the family of pro-apoptotic proteins TRAIL and related mutants, in particular KillerTRAIL, are bound and exposed on the external surface of the obtained liposomes.

The differences between the two preparation methods consist in the fact that the first preparation method, that is the one related to the conjugation of the pro-apoptotic TRAIL proteins and related mutants, in particular KillerTRAIL, on the surface of the preformed vesicles through a *linker* such as a maleimide (MAL) linker at the end of the phospholipid-PEG derivative, preferably distearoyl-phosphatidyl-ethanolamine-polyethylene glycol (DSPE-PEG), allows to increase the number of KillerTRAIL molecules that can interact with the receptor sites present on cells and tissues target.

The second method partially reduces, without compromising the therapeutic function of the pro-apoptotic protein, the number of KillerTRAIL molecules on the surface, due to a partial localization of the therapeutic protein within the vesicular structure, even with its subsequent release from the liposomal formulations after interaction with therapeutic targets, as a consequence of the preformed synthesis of the phospholipid derivative, preferably DSPE-PEG-KillerTRAIL.

In the first method, the synthesis strategy was to form liposomal vesicles consisting of different lipids, preferably a derivative of phosphatidylcholine, more particularly, a derivative of dipalmitoyl-phosphatidylcholine (DPPC), a derivative of cholesterol (Chol), a phospholipid-PEG, preferably distearoyl-phosphatidyl-ethanolamine-N-maleimide-polyethylene glycol (DSPE-PEG-maleimide), at different molar fractions, preferably 6/3/1, using different preparation methods. A preferred method was the evaporation method of the lipid film on a thin layer or Thin Layer Evaporation (TLE), coupled with the method of extrusion through polycarbonate filters with different sizes, preferably 400 and 200 nm, through a stainless steel extruder (Lipex Biomembranes, Northern Lipids Inc., Vancouver, BC, Canada).

The TRAIL protein or its mutant, in particular KillerTRAIL, was initially purified by dialysis (regenerated cellulose cut-off 10,000 Da) to remove the solution in which the protein was stored (Salts, ethylenediaminetetraacetic acid EDTA, dithiothreitol DTT) in

which it is marketed. Subsequently, a treatment of the protein solution with only 8 mM DTT was carried out at room temperature for 1 hour in order to avoid the spontaneous formation of disulfide bridges that would lead to the formation of the protein in the form of a trimer. To maintain the optimal conditions for the production of the conjugate, the protein solution from the DTT was further purified by means of a desalting chromatographic column. Under these experimental conditions the KillerTRAIL protein is found in monomeric form.

The KillerTRAIL protein, in monometric form, was conjugated using the thiol group (–SH) of the amino acid cysteine in position 230, to the obtained liposomes which have on their surface the phospholipid-PEG, preferably distearoyl-phosphatidyl-ethanolamine-N-maleimide -polyethylene glycol (DSPE-PEG-maleimide or DSPE-PEG-MAL), exploiting the conjugation strategy between the TRAIL protein or one of its mutants, in particular KillerTRAIL, and the DSPE-PEG-maleimide (DSPE-PEG-MAL).

In the second method, the synthesis strategy was to conjugate the TRAIL protein or one of its mutants, in particular KillerTRAIL, using the thiol group (-SH) of the amino acid cysteine in position 230, with the phospholipid-PEG, preferably distearoil -phosphatidyl-ethanolamine-N-maleimide-polyethylene glycol (DSPE-PEG-maleimide or DSPE-PEG-MAL). The phospholipid-PEG-TRAIL conjugate was then used to make a liposomal vector.

Both synthesis processes do not modify the therapeutic activity of the native protein either *in vitro* or *in vivo*. Conjugation is obtained by bonding on a polymeric backbone of the polyethylene glycol (PEG) type, with a size ranging from 2-200 kDa, preferably 10-50 kDa. Conjugation is carried out on the PEG-KillerTRAIL adduct and the phospholipid-PEG-KillerTRAIL conjugate is then used to make a liposomal vector whose formulation and preparation is described below. Preferably the PEG is a PEG of about 2 kDa.

The vector obtained (Lipo-PEG-protein, preferably Lipo-PEG-KillerTRAIL) has amphiphilic characteristics that can be exploited to synergistically convey hydrophilic, lipophilic and amphipathic drugs in the therapeutically active derivative, while maintaining both the targeting and the therapeutic activity of the protein unaltered. In fact, the lipophilic portion of the conjugate is inserted into the liposomal *bilayer*, anchoring itself to the phospholipid bilayer which in itself is capable of encapsulating / complexing lipophilic drugs, based on the molecular weight and chemical-physical parameters. At the same time, the lipophilic portion of the derivative, anchored by the phospholipid bilayer, surrounds one or more aqueous compartments of the liposomal vesicle that are able to encapsulate / complex hydrophilic drugs based on their molecular weight and their chemical-physical properties. The lipophilic component (*lipid* bilayer) and the hydrophilic component (aqueous compartment) of the liposomal vesicles can also encapsulate / complex amphipathic drugs, depending on their molecule weight and their chemical-physical properties, in which the lipophilic portion of the drug is inserted in the phospholipid *bilayer*, while the hydrophilic portion of the drug is inserted in the aqueous core of the liposomes.

In all types of encapsulation / complexation of drugs inside the liposomal vesicles described here, the PEG chains and the protein are organized towards the aqueous environment, forming an external polymeric coating of the liposomal vesicles which have the targeting portion of the protein on the external surface. pro-apoptotic which maintains its therapeutic activity.

The proteins of the TRAIL family and related mutants, such as KillerTRAIL, to which the invention refers are those which have only one cysteine in the amino acid chain. This allows to obtain a specific conjugation between the -SH group of the native protein and the spacer or *linker*. In particular, to obtain this conjugation the maleimide

group (MAL) is used by exploiting the Michael reaction [57] which favors the formation of the very stable chemical bond CS.

Another object of the invention is the identification of the sequence of the KillerTRAIL protein used for the preparation of the Lipo-PEG-protein vector, preferably Lipo-PEG-KillerTRAIL object of the invention.

Another object of the invention are the pharmaceutical compositions containing the Lipo-PEG-protein vector, preferably Lipo-PEG-KillerTRAIL, object of the invention and the excipients pharmaceutically used for their preparation.

Still another object are pharmaceutical preparations for diagnostic use comprising the liposomal system object of the invention and a diagnostic agent.

Further objects of the invention are the applications for therapeutic and diagnostic use of the above obtained liposomal formulations.

Further objects, objects and advantages will become apparent from the detailed description of the invention.

Brief description of the figures

Figure 1: HPLC chromatogram of the KillerTRAIL protein.

Figure 2: ESI MS spectrum of the KillerTRAIL protein; Panel A: molecular weight of the protein obtained following the deconvolution process applied to the mass spectra reported in panels B and C. Panel B: mass spectrum with high instrumental signal (TIC, Total Ion Current); Panel C: mass spectrum of the instrumental low-signal protein (TIC, Total Ion Current).

Figure 3: MALDI-TOF / TOF fragmentation spectrum of the peptide with m / z 2549.19.

Figure 4: Panel A: TIC of the extracted ion of the double and triple charged ions corresponding to thepeptide *linker*. Panel B: Peptide fragmentation spectrum with m / z 2549.19.

Figure 5: Sequence coverage of KillerTRAIL Protein (soluble) (human), (recombinant).

Figure 6: Killer peptide sequence of the KillerTRAIL protein.

Figure 7: Schematic of the post-conjugation method of the KillerTRAIL protein on the surface of the liposomal system.

Figure 8: Chromatographic analysis of nanosystems containing KillerTRAIL on the surface of the liposomal vesicles.

Figure 9: Pharmacokinetic profiles of KillerTRAIL and Lipo-PEG-KillerTRAIL. Experiment performed *in vivo* on rat mouse models (n = 5; 10 mg / rat, iv); the blood concentration was evaluated by analysis with an ELISA assay.

Figure 10: Antitumor activity *in vivo* on mouse models of the different formulations: control (untreated or white animal), KillerTRAIL, and Lipo-PEG-KillerTRAIL in mouse models (Balb / c mice) xenographed with HCT- colon cancer tumor cells 116. The different compounds were administered every 4 days through the tail vein of the aforementioned mouse models (100 µg / mouse[P1] [MVP2]) (n = 5; * $p < 0.05$).

Figure 11: MALDI-TOF-MS spectrum of DSPE-PEG-maleimide (MAL) 2 kDa (A) and of the derivative DSPE-PEG-KillerTRAIL (B).

Figure 12: SDS-PAGE of reactions with PEG (20 kDa) (A) and DSPE-PEG-maleimide (MAL) (2 kDa) (B), in the presence of a reducing and non-reducing agent. KillerTRAIL was used as a standard reference sample.

Figure 13: antitumor activity *in vivo* of KillerTRAIL and its phospholipid derivative-PEG-KllerTRAIL.

Figure 14: Comparison of the antitumor activity *in vivo* of KillerTRAIL, PEG-KillerTRAIL and the phospholipid-PEG-KillerTRAIL derivative.

Figure 15: Biodistribution in different organs and biological fluids (blood, liver, kidney, pancreas, lungs, urine) of KillerTRAIL (A), and phospholipid-PEGKillerTRAIL (B) in mice xenografted with HCT116 colon cancer cells. Protein concentration, identified in blood,

liver and tumor mass at different incubation times (15, 30, 60, 24 min.) For KillerTRAIL and phospholipid-PEG-KillerTRAIL (C).

Detailed description of the invention

Within the scope of the present invention:

- The terms polyethylene glycol and PEG are to be considered synonyms and will be used indifferently one with respect to the other;
- The term TRAIL will be used to identify the KillerTRAIL protein, a pro-apoptotic protein of exogenous type, with monomeric structure obtained by a process *ad hoc* described in the invention, belonging to the family of *tumor necrosis factor* cytokines, the proteins of the family are: TNFSF10, TL2; APO2L; CD253; Apo-2L [19,20] and the term TRAIL will be used to indicate indifferently the KillerTRAIL protein and any of the above proteins also with monomeric structure;
- The term *linker* identifies the connecting element between the polyethylene glycol (PEG) and the pro-apoptotic protein (TRAIL) as specified in the following of the present description, the maleimide (also called MAL) *linker* being preferred;
- The term "adduct" identifies a compound consisting of a phospholipid linked to a polyethylene glycol or PEG, also referred to as phospholipid-PEG, preferably DSPE-PEG;
- The term "derivative" or PEG-TRAIL or also PEG-*linker*-TRAIL identifies a compound consisting of polyethylene glycol or PEG and TRAIL as defined above;
- The wording phospholipid-PEG-protein or phospholipid-PEG-TRAIL, in particular phospholipid-PEG-KillerTRAIL, can be identified indifferently with the terms "conjugate" or "polymeric conjugate" and always provides that the

PEG is bound to one end of the phospholipid and at the opposite end to the monometric TRAIL protein, in particular KillerTRAIL, with a covalent CS bond on the cysteine residue of the sequence of said protein through a *linker*, such as for example the maleimide group (MAL);

- The wording "vector" or "nanovector" or Lipo-PEG-TRAIL or Lipo-PEG-protein identifies a liposome that hasintegrated in the *bilayer* the phospholipid-PEG-TRAIL conjugateor lipid bilayer, in particular the DSPE-PEG-KillerTRAIL conjugate, and the wording Lipo-PEG-KillerTRAIL indicates the liposomal system which has the KillerTRAIL protein in monomeric form exposed on the liposomal surface.

In the context of the present invention, the term heterobifunctional polyethylene glycol (PEG) means a polyethylene glycol conjugated at one end with a phospholipid, such as DSPE (distearoyl-phosphatidyl-ethanolamine), and at the other end linked with a group reactive towards the thiol group of the protein, for example the maleimide group or MAL (therefore the wording PEG, unless otherwise specified, will always be considered linked, at one end, to the *linker* or reactive group towards the thiol group of the protein). The PEG derivatives used in the invention are commercial products (https://avantilipids.com/product-category/polymers-polymerizable-lipids) and, typically, the PEG derivatives that can be used in the present invention are non homogeneous marketed on the basis of average molecular weight. For example, commercially available PEG 2,000 and 5,000 Da derivatives (https://avantilipids.com/product-category/polymers-polymerizable-lipids) typically contain polyethylene glycol chains that may vary slightly from the indicated molecular weight on the packaging of the commercial product and have a *standard* deviation equal to ± 500 Da. The molecular weight of the PEG is preferably in the range 2-200 kDa, preferably 10-50 kDa.

In the context of the present invention, the term Lipo-PEG-KillerTRAIL means a liposomal system, i.e. a nanovector with a vesicular structure formed by an internal aqueous compartment or core, and / or aqueous interlamellar spaces, or aqueous compartments between a lipid double layer or *bilayer* and the next one, and one or more lipid double layers or *bilayerr*, in which the phospholipid-PEG-protein is inserted, more precisely the phospholipid-PEG-*linker*-protein, which has a derivative exposed on the liposomal surface belonging to the TRAIL therapeutic protein family, in particular KillerTRAIL.

Still in the context of the present invention, the "conjugate" or "polymeric conjugate" identifies the phospholipid-PEG-protein or phospholipid-PEG-TRAIL, in particular phospholipid-PEG-KillerTRAIL, which constitutes an amphipathic system or lipid *backbone*, more precisely the phospholipid-PEG-*linker*-protein (or phospholipid-PEG-*linker*-TRAIL or phospholipid-PEG-*linker*-KillerTRAIL), which can generate, at its critical micellar concentration, stable micellar forms containing on the surface the PEG chains conjugated to the protein therapeutic TRAIL, in particular KillerTRAIL. The phospholipid-PEG-KillerTRAIL micellar conjugates, more specifically DSPE-PEG-KillerTRAIL, have a different structure than the liposomal vectors Lipo-PEG-KillerTRAIL and are formed by a hydrophobic core consisting of phospholipids, more specifically DSPE, capable of encapsulating / complexing lipophilic drugs on the basis of their chemical-physical characteristics, surrounded on its external surface by PEG chains that always expose the therapeutic protein TRAIL, more particularly KillerTRAIL, on the external surface. Such polymeric conjugates, which naturally form under the reaction conditions described above, can also be present, generally in negligible quantities, with the liposomal vesicles Lipo-PEG-KillerTRAIL.

The reference critical micellar concentration, often abbreviated as CMC, is a parameter that represents the concentration value of a surfactant or phospholipid-PEG

suspension at which, once thereached or exceeded *Krafft* temperature (critical micellar temperature) is, a certain number of monomers aggregate leading to the formation of micelles. This parameter is a known parameter and can be easily identified by a person skilled in the art based on his knowledge combined with the information of the present description.

In the context of the present invention with the wording TRAIL and its variants or TRAIL, and its mutants, we mean theprotein TRAIL(also known as TNFSF10, TL2; APO2L; CD253; Apo-2L) which is a member of the ligand family of TNF.

Within the scope of the present invention, the protein is in monomeric form and is selected from LTα, KillerTRAILβ, LTβ, the CD20 ligands; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), KillerTRAIL, TNF-apoptosis induced ligand (KillerTRAIL), TNFSF10, TL2; APO2L; CD253; Apo-2L (Entrez Gene ID: 8743; access number NM_003810.2; UniProtKB / Swiss-Prot: P50591; UniProtKB / TrEMBL: Q6IBA9).

A particularly preferred TRAIL mutant is the protein marketed as KillerTRAIL.

The present invention relates to the delivery by means of polymeric conjugation of pro-apoptotic therapeutic proteins of the exogenous type, in particular the cytokine CD523 or TRAIL and its mutants. Among the various cytokines belonging to the superfamily of *tumor necrosis factor* cytokines, the cytokine containing awas selected as particularly preferred peptide *linker* (KillerTRAIL or k-TRAIL ™) which makes its tertiary structure more stable. This cytokine, like its homologs, induces apoptosis upon binding to pro-apoptotic transmemban receptors.

Due to the toxicity problems (hepatic) and low half-life of these cytokines, the aim achieved by the present invention was to reduce these problems through a conjugation on a polymeric *backbone*.

The object of the present invention is therefore a method for obtaining polymeric conjugates of the cytokine CD523 or TRAIL and its modifications, in particular KillerTRAIL.

Advantageously, the conjugates are monoconjugates, in which the functionalization of the protein has been performed at the level of a selective cysteine site. In fact, the main characteristic of TRAIL is that of having only one cysteine site and this determines a more site-specific type of bond, since generally the cysteines (and therefore the thiol groups) are in reduced quantities compared to the other amino acids and in this specific case the KillerTRAIL has only one. This configuration, in fact, favors the unidirectional and selective conjugation with the PEG-maleimide and avoids the random bonds on endogenous proteins, the selective conjugation of the protein to the PEG chains, preventing their aggregation, and confirming the steric hindrance of the protein to form trimers.

The conjugation reaction is carried out in an aqueous environment allowing the selective binding of a functionalized polyethylene glycol at one end with a phospholipid and at the opposite end with a *linker* or reactive group, selective for the thiol of the cysteine present in the peptide chain of the protein.

Preferably the reactive group of the phospholipid-PEG is a maleimide group.

Preferably the protein is TRAIL, more preferably the protein is k-TRAIL and the cysteine involved in the derivatization is in position 230 of the peptide chain. The presence of a single cysteine in position 230, which is mnaturally present in the amino acid chain in this family of cytokines, allows the selective PEGylation of only one active site of the copolymer. This type of synthetic process allows to reduce the steric hindrance of the endogenous protein and therefore favor its interaction with the receptor site, without modifying its link with the receptor and consequently its therapeutic activity. Furthermore, the PEGylation of the cysteine alone present in the side chain of the

protein, initially not recommended according to the data reported in the literature, unexpectedly and advantageously improved the therapeutic action of the native protein without interfering with its targeting effect.

Variant or mutant means that the TRAIL protein differs in at least one amino acid position from theTRAIL protein *wild type* (also known as TNFSF10, TL2; APO2L; CD253; Apo-2L) (Entrez Gene ID: 8743; access number NM_003810. 2; UniProtKB / Swiss-Prot: P50591; UniProtKB / TrEMBL: Q6IBA9).

A particularly preferred mutant of TRAIL is the protein present on the market as KillerTRAIL and in particular its monomeric form obtained using an ad hoc process described in the invention and which has the following sequence resolved by tandem mass spectrometry (MS / MS) and a method of mass mapping based on ion strategy extract and is shown below: specific sequence of TRAIL (Seq ID No 1: **TSEE**TISTVQ EKQQNISPLV RERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG) conjugated via the head residues Seq ID No 4: **TSEE** to the specific sequence of the Killer peptide (Seq ID No 2: HHHHHHYLMYGTVTLGY**TSEE**). Therefore, the complete sequence of KillerTRAIL is the Seq ID No 3: HHHHHHYLMYGTVTLGYTSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSN TLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRF QEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELK ENDRIFVSVTNEHLIDMDHEASFFGAFLVG.

The present invention aims to overcome the critical problems typical of therapeutic proteins such as rapid elimination, degradation and toxic accumulation (formation of

immune complexes), site-specific direction and co-administration of active ingredients with a therapeutic activity synergistic for a specific pathology.

The present invention therefore relates to the conveyance of pro-apoptotic therapeutic proteins of the exogenous type, in particular KillerTRAIL and the cytokine CD523 or TRAIL and its mutants in monomeric form, conveyance achieved through the realization of liposomal type nanovectors obtained by inserting it into the double liposomal orlipid layer *bilayer* of phospholipid-PEG-TRAIL liposomes, preferably phospholipid-PEG-KillerTRAIL, in particular DSPE-PEG-KillerTRAIL, in which the therapeutic protein of the TRAIL protein family in monomeric form, and in particular KillerTRAIL, will be arranged in whole or in part on the external surface of the liposomal vesicles, thus forming liposome nanovectors with the protein object of the invention having a therapeutic and targeting activity.

Among the various cytokines belonging to the superfamily of *tumor necrosis factor* cytokines, the cytokine containing ahas been selected as particularly preferred, peptide *linker* (KillerTRAIL) making its tertiary structure more stable. This cytokine, like its homologs, induces apoptosis upon binding to pro-apoptotic transmemban receptors.

Due to the toxicity problems (at the liver tissue level) and low half-life of these cytokines, the aim achieved by the present invention was to reduce these problems through the reduction of the TRAIL protein, more particularly KillerTRAIL from the trimeric to the monomeric form and its conjugation on the polymeric *backbone* and obtaining liposomal vectors.

The object of the present invention is therefore a method for obtaining liposomal vesicles which have on the surface the polymeric conjugates of the cytokine CD523 or TRAIL and its mutants, in particular KillerTRAIL, together with the therapeutic, diagnostic and theranostic uses of said therapeutic and targeting liposomal vectors.

The presence of only one cysteine, naturally present in the amino acid chain in this family of cytokines, allows the selective PEGhylation of only one active site of the copolymer. This type of synthetic process allows to reduce the steric hindrance of the endogenous protein and therefore favor its interaction with the receptor site, without modifying its link with the receptor and consequently its therapeutic activity. Furthermore, the PEGylation of the cysteine alone present in the side chain of the protein, initially not recommended according to the data reported in the literature, unexpectedly and advantageously improved the therapeutic action of the native protein without interfering with its targeting effect.

Preferably the protein is TRAIL, more preferably the protein is KillerTRAIL and the cysteine involved in the conjugation process with the PEG is in position 230 of the peptide chain.

The TRAIL presents the sequence shown in the list posted on the site https://www.uniprot.org/uniprot/P50591 aa 95-281 and comprises the sequence: Seq ID No 1:

TSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKIN SWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYI YKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMD HEASFFGAFLVG.

The KillerTRAIL presents the specific sequence of TRAIL: Seq ID No 1: **TSEE**TISTVQ EKQQNISPLV RERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG conjugated via the head residues Seq ID No 4: **TSEE** to the specific sequence of the peptide Killer (Seq ID No 2: HHHHHHYLMYGTVTLGY). Therefore, the complete sequence of KillerTRAIL is the Seq

ID No 3:
HHHHHHYLMYGTVTLGYTSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSN
TLSSPNSKNEKALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRF
QEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELK
ENDRIFVSVTNEHLIDMDHEASFFGAFLVG.

Mutants having a homology of 50%, 52%, 54%, 56%, 58%, 60%, 62%, 64%, 66%, 68%, 70%, 72 are included in the scope of the invention. %, 74%, 76%, 78%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98%, 99% or greater sequence homology than the TRAIL protein or KillerTRAIL or comprising a polypeptide with at least 50%, 52%, 54%, 56%, 58%, 60%, 62%, 64%, 66%, 68%, 70%, 72%, 74 %, 76%, 78%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98%, 99% or greater sequence similarity than SEQ polypeptides ID No 1, Seq ID No 3 and SEQ ID No 2 and to the specific sequence of the KillerTRAIL SEQ ID No 3.

The invention is based on the choice of specific reaction conditions and on the use of a modified PEG having a phospholipid and on the other end a reactive group for the cysteine thiol present in the amino acid sequence of the protein (for example maleimide (MAL), preferably N-alkyl-maleimide, such as for example N-methyl-maleimide or N-ethyl-mal eimide) so as to selectively react with the thiol group of the amino acid sequence.

The SH- thiol reactive group can be selected from, but is not limited to: halo-acetyls, maleimides, phthalimides, aziridines, acryloyls, arylating agents, vinyl sulfones, pyridyl disulphides, TNB-thiols and bridge reducing agents SS. Other imides on the market such as n-hydroxy-succinimide, are not selective towards thiols and also react with amines. Preferably it can be: DSPE-PEG-maleimide 10,000 Da, DSPE-PEG-maleimide 20,000 Da, DSPE-PEG-maleimide 40,000 Da, DSPE-PEG-maleimide branched 10,000 Da, DSPE-PEG-maleimide branched 20,000 Da and DSPE-PEG-

maleimide branched 40,000 Da and / or their mixture, other polymers having the same properties of PEG like polyvinylpyrrolidone; polyacryloyl morpholin; N-hydroxy-eyil-methacrylamide copolymer; poly (2-etyl-osxazolyne); poly-glutammic acid; hyaluronic acid; polysialic acid; co-polymers of PEG-poly-aminoacidi, phospholipids-PEG with thiol groups, PEG-maleimide (PEG-MAL) (Biochempeg Worldwide PEG Supplier https://www.biochempeg.com/Maleimide-PEG? Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids), PEG-orthopyridine disulfide (PEG-OPSS) (Creative PEGWorks, https://www.creativepegworks.com/product.php?cid = 8; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids), PEG iodine-acetamide (PEG-IA) (NOF American corporation, https://www.nofamerica.com/store /index.php?dispatch=categories.view&category_id=5; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids) and PEG-vinyl sulfone (PEG-VS) (JenKem Technology USA, https : //www.jenkemusa.com/products; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids).

More specifically, the present invention relates to the development of a PEGylated liposomal structure delivery system for the Killer TNF-apoptosis induced ligand (KillerTRAIL). The conjugation strategy that has been evaluated concerns the possibility of binding the TRAIL protein, preferably KillerTRAIL, to the activated phospholipid-polyethylene glycol, and integrated into the liposomal *bilayer*, thanks to the presence of the thiol group (-SH) of cysteine in position 230 of the protein chain.

Other PEG derivatives have also been developed having specific reactivity towards the thiol group. In addition to PEG-maleimide (PEG-MAL), other functional groups can be mentioned that have the same reactive (nucleophilic substitution reaction) towards thiols, such as PEG-ortho-pyridine disulfide (PEG-OPSS), PEG iodine-

acetamide (PEG-IA) and PEG-vinyl-sulfone (PEG-VS). Currently, PEG-MAL and PEG-OPSS are the most widely used derivatives.

Conjugation is obtained by binding with a phospholipid-PEG (the exhaustive list of which is given below), preferably distearoyl-phosphatidyl-ethanolamine-polyethylene glycol (DSPE-PEG) derivatized with a reactive group towards thiols, preferably a maleimide. The molecular weight of the PEG is preferably in the range 2-200 kDa, preferably 10-50 kDa.

However, other groups known to be reactive for the polymer conjugation of proteins, reactive towards thiols such as: halo-acetyls, maleimides, phthalimides, aziridines, acryloyls, arylating agents, vinyl-sulfones, pyridyl-disulfides, TNB-thiols and bridges reduction agents SS. Other imides on the market such as n-hydroxy-succinimide, are not selective towards thiols and also react with amines.

The most preferred polyethylene glycols are DSPE-PEG-maleimide 10,000 Da, DSPE-PEG-maleimide 20,000 Da, DSPE-PEG-maleimide 40,000 Da, DSPE-PEG-maleimide branched 10,000 Da, DSPE-PEG-maleimide branched 20,000 Da and DSPE-PEG - branched maleimide 40,000 Da and related mixtures.

Similarly, other polymers with analogous action can also be used, as the export of the branch is able to understand and which can be chosen from: polyvinylpyrrolidone; poly-acryloyl-morpholine; N-hydroxy-ethyl-methacrylamide copolymer; poly (2-ethyl-oxazoline); poly-glutamic acid; hyaluronic acid; poly-sialic acid; and PEG-poly-amino acid copolymers. However, PEG is preferred as it is a monofunctional polymer. Conjugation of this protein can also be obtained through the other polymers indicated above, but the reaction yield with respect to PEG would be lower. In addition, PEG is a biocompatible and biodegradable copolymer approved by the *European Medicine Agency* (EMA) and the *Food and Drug Administration* (FDA).

As will be clear from the preparation methods indicated below, the PEG chain used is anchored to the liposomal system thanks to the presence in the *bilayer* of a phospholipid-PEGylate. While the phospholipid by its nature will insert itself in the *bilayer*, and the PEG chain will be exposed to the outside and the exposed end will contain the reactive maleimide group (MAL) to which to conjugate the therapeutic protein belonging to the TRAIL family, in monomeric form, more specifically KillerTRAIL. The system thus produced was characterized from a chemical-physical and biological point of view.

The conjugation can be carried out on the surface of the liposomal vector whose formulation and preparation is described below. One of the strategies proposed for the delivery of the TRAIL protein is its conjugation on the surface of the liposomal vesicles, according to the strategy described above, to form targeting and therapeutic liposomal systems.

In this regard, an attempt was made to insert the macromolecular conjugates of TRAIL into liposomal systems so that the protein is directed at least in part towards the external surface of the *bilayer*, remaining available for receptor recognition.

A preferred liposomal formulation, used for post-conjugation tests, consists of phospholipid: Chol: phospholipid-PEG with various molar ratios ranging from 0.1-6 for phospholipid, 0.1-3 for cholesterol and 0.01-3 for the phospholipid-PEG.

Among the phospholipids present on the market, it is possible to use the following ones.

Among the phospholipid-PEG adducts it is possible to use adducts containing functional groups reactive towards thiols, such as: PEG-maleimide (PEG-MAL) (Biochempeg Worldwide PEG Supplier https://www.biochempeg.com/Maleimide-PEG?gclid=CjwKCAjwhOD0BRAQEiwAK7JHmGONpdU0ENqiuf-qaZfnDw3PKMSfpamF3LYsypDYqunIEuHalipeqBoC3rAQAvD_BwE; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids), orthopyridine

PEG-disulfide (PEG-OPSS) (Creative PEGWorks, https://www.creativepegworks.com/product.php?cid=8; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids), PEG iodine-acetamide (PEG-IA) (NOF American corporation, https://www.nofamerica.com/store/index.php?dispatch=categories.view&category_id=5 ; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids) and PEG-vinyl sulfone (PEG-VS) (JenKem Technology USA, https://www.jenkemusa.com/products; Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids).

Among these, the PEG-MAL was chosen which among all guarantees a stable and non-cleavable bond *in vivo* in the presence of biological fluids or natural and synthetic *buffers*. The phospholipids generally bound to PEG usable in this invention are listed in the description of the invention.

The process for obtaining the nanovectors of the invention comprises the following stages (unless otherwise indicated, the reaction stages are carried out at ambient temperature and pressure):

(i) Solubilize in an appropriate organic solvent an aliquot of one or more phospholipids selected from neutral, cationic and anionic and add an aliquot of a PEG functionalized phospholipid and chosen from: phospholipid-PEG (generally available on the market) and phospholipid-PEG -protein (previously prepared), the phospholipid functionalized with PEG being in relationship with the one or more phospholipids according to the quantity chosen in the molar fraction range 0:10 - 10: 0.

In particular, the phospholipid can be DSPE and the protein can be KillerTRAIL in order to have the DSPE-PEG-KillerTRAIL conjugate.

The one or more phospholipids can be selected from neutral, cationic and / or anionic, possibly in combination with an aliquot of cholesterol.

The organic solvent can be selected from chloroform, ether, methanol or mixtures of these solvents, present in a ratio of between 10-0.1 and 0.1-10 in molar fraction with respect to one or more phospholipids.

The phospholipid-PEG-protein conjugate will be present in a ratio between 10-0.1 and 0.1-10 in molar fraction with respect to one or more phospholipids.

(ii) Dry the mixture of step (i), for example by eliminating the organic solvent under a continuous nitrogen flow at a temperature between 18 and 75°C;

(iii) then rehydrate with water or an aqueous solution of phosphate buffer around neutrality, generally pH 5-8, typically around pH 7.4, by mechanically stirring the aqueous suspension, for example using a *vortex-mixing*, i.e. an orbiting mechanical stirrer, for a number of cycles between 1 and 10 times, for a time between 30 seconds and 15 minutes; preferably alternate mechanical stirring (from a minimum of 3 to a maximum of 20 cycles) at room temperature by *vortexing-mixing* with heating in a water bath (from a minimum of 3 to a maximum of 20 cycles) at a temperature interval between 18 and 75°C, for a time between 30 seconds and 15 minutes;

(iv) Extrude the liposomes obtained in step (iii), for example using the following preferred conditions: through polycarbonate filters with dimensions between 30 nm and 1 µm (according to the manufacturing specifications reported by the manufacturer, https://www.fishersci.it/it/it/brands/IGM1SIIB/whatman.html) and for a number of passages between 1 and 30, at a temperature between 18 and 75°C, at a pressure between 10 and 700 mBar, obtaining the corresponding liposomes.

A further freeze-drying step can be provided.

A) If a PEG-functionalized phospholipid not containing TRAIL is used to obtain the liposomes of stage (iv), these liposomes are then conjugated with the TRAIL protein, in monomeric form, or one of its mutants, in particular KillerTRAIL, by means of the thiol group (–SH) of the amino acid cysteine in position 230. Conjugation is carried out on the *linker* of the PEG functionalized phospholipids, preferably distearoyl-phosphatidyl-ethanolamine-N-maleimide-polyethylene glycol (DSPE-PEG-maleimide), present on the surface of liposomes obtained in step (iv) after extrusion.

The conjugation strategy between the TRAIL protein in monomeric form, or one of its mutants, in particular KillerTRAIL, and the PEG-functionalized phospholipid includes the stages of:

- Preliminarily purify the TRAIL protein, to obtain it in monomeric form, or its mutant, in particular KillerTRAIL, by dialysis in phosphate buffer (typically PBS at pH 7.4) in the presence of 0.05 mM EDTA (typically dialysis membranes with *cut-off* of 2,000 Da) to eliminate the dithiothreitol (DTT) used in the protein storage solution itself. The purity of the purified sample can be subsequently analyzed by UV / Vis spectroscopy and *Reverse Phase High Performance Liquid Chromatography* (RP-HPLC). Under these experimental conditions the KillerTRAIL protein is found inform **monomeric**;
- The nano-vector prepared in step (vi) in a molar ratio 1: 4 (protein: liposome) is added to the buffer solution containing the TRAIL protein or its mutant, in particular KillerTRAIL (typically concentrations of 1 mg / mL). The reaction mixture is kept under constant stirring at room temperature until the reaction is complete. The progress of the reaction can be monitored by taking an aliquot (25 µL) of sample at different incubation times for chromatographic analysis;

- Once the reaction is complete, monitored thanks to RP-HPLC (Example 5, Figure 8) and the electrophoretic technique SDS-PAGE (Example 8, Figure 14), the reaction product is purified again using a dialysis membrane (*cut-off* 30,000 Da) to eliminate the residual amount of protein that has not reacted during the synthesis of the compound In this way, the nanovectors of the invention Lipo-PEG-KillerTRAIL are obtained.

B) In the event that a PEG-functionalized phospholipid containing TRAIL, more particularly KillerTRAIL, that is the phospholipid-PEG-protein conjugate, has been used to obtain the liposomes of stage (iv), said conjugate will have been previously prepared with a method comprising the following steps main:

- Preliminarily purify the TRAIL protein or its mutant, in particular KillerTRAIL, by dialysis in phosphate buffer (typically PBS at pH 7.4) in the presence of 0.05 mM EDTA (typically dialysis membranes with a *cut-off* of 2,000 Da are used) to eliminate the dithiothreitol (DTT) used in the storage solution of the protein itself. The purity of the purified sample can be subsequently analyzed by UV / Vis spectroscopy and *Reverse Phase High Performance Liquid Chromatography* (RP-HPLC). Under these experimental conditions the KillerTRAIL protein is found inform **monomeric**;
- Theis added to the buffer solution containing the TRAIL protein or its mutant, in particular KillerTRAIL, (typically concentrations of 1 mg / mL).*linker,* phospholipid-PEG-preferably the phospholipid-PEG-maleimide, more preferably DSPE-PEG-maleimide,, in molar ratio 1: 4 (protein: PEG). The reaction mixture is kept under constant stirring at room temperature until the reaction is complete. The progress of the reaction can be monitored by taking an aliquot (25 µL) of sample at different incubation times for chromatographic analysis;

- Once the reaction is complete, monitored thanks to RP-HPLC (Example 3, Figure 10) and the electrophoretic technique SDS-PAGE (Example 10, Figure 12), the reaction product is purified again using a dialysis membrane (*cut-off* 30,000 Da) to eliminate the residual amount of protein that did not react during the synthesis of the compound.

C) According to an alternative of the process, the nanovectors of the invention can be prepared starting from stage (i) up to stage (iv) without adding either the phospholipid-PEG adduct or the phospholipid-PEG-protein conjugate. Then prepare the phospholipid-PEG-protein conjugate separately as indicated in section B) and continue with the process steps indicated below:

- Disperse the phospholipid-PEG-KillerTRAIL conjugate, more specifically DSPE-PEG-KillerTRAIL, in an isotonic solution at physiological pH, such as phosphate buffer, water, 0.9% (w / v) physiological solution, 5% (w / v) glucose solution, to favor the formation of a nano-aggregate with a micellar structure; purify (using dialysis membranes with a *cut-off* of 50 kDa, Spectra / Por, dialysis membrane tubing, Fiscer Scientific Italia, Rodano (MI), Italy) then the nanoaggregates thus obtained and if necessary, proceed with further processes purification, from a minimum of 2 to a maximum of 10;
- Mix the liposomes of the previous step (iv) with the newly prepared nanoaggregates; the mixing will take place under mechanical stirring and at a temperature between 18 and 75°C, typically magnetic and thermal plates are used and speeds between 10 and 1000 rotations per minute (rpm) with times between 10 minutes and 24 hours; the phospholipid-PEG-protein conjugate micelles, more particularly DSPE-PEG-KillerTRAIL micelles, are added in an aliquot between 0.1 and 4, in molar fraction with respect to the liposomes obtained in step (iv).

The mixing process is carried out in the temperature range from 18-75°C, which corresponds to a temperature range higher than the transition temperature Tm of the phospholipids used to prepare the lipid film and obtain the liposomes object of the invention.

In particular, by transition temperature or Tm we mean a temperature, characteristic for each phospholipid, below which the double state or *bilayer* obtained is in a gel phase (rigid structure) and above which the double layer or *bilayer* obtained is in a liquid-crystalline phase (more fluid structure). Tm is an important parameter known to those skilled in the art who use it to characterize liposomal vectors from a chemical-physical point of view and in particular it indicates the state of rigidity and / or fluidity of the phospholipid *bilayer* which is able to modify the rigidity of the *bilayer*, the encapsulation of hydrophilic, lipophilic and amphipathic drugs, and their release kinetics. Tm is measured using the differential scanning calorimetry (DSC) method.

In this case, the heating of the liposomes to a temperature higher than the transition temperatures or Tm of the phospholipids allows to increase the fluidity of the phospholipid *bilayer* and favors the insertion of the micellar conjugates of phospholipid-PEG-protein, inside the lipid *bilayer* of liposomes with the therapeutic protein TRAIL, in monomeric form, (KillerTRAIL) present on the external surface of liposomes.

The nanovectors of the invention thus produced and purified (Lipo-PEG-TRAIL) can be lyophilized and stored in the temperature range between 0 and -32°C, more preferably at the temperature of -20°C for subsequent uses.

The nanovectors thus produced and purified can be administered as they are or, in the event that it is chosen to co-administer them synergistically with other lipophilic, hydrophilic or amphiphilic drugs, the drugs will be added as indicated below. The indicated methodologies, combined with the teachings of the present invention, are within the reach of the person skilled in the art.

If it is intended to combine the nanovectors of the invention with lipophilic drugs, aliquots of one or more lipophilic drugs can be added in the preparation step which involves the use of organic solvents, typically step (i), during the dissolution step of the neutral, cationic and / or anionic lipids and / or their combinations.

If it is intended to combine the nanovectors of the invention with hydrophilic drugs, aliquots of one or more hydrophilic drugs can be added in the preparation step which involves rehydration in water or in the aqueous solution consisting of a buffer or *buffer*, typically step (iii), during the hydration phase of the lipid vesicles.

If it is intended to combine the nanovectors of the invention with amphipathic drugs, aliquots of one or more amphipathic drugs can be added in the preparation step which involves the use of organic solvents, typically step (i), during the dissolution step of the neutral, cationic and / or anionic lipids and / or their combinations and / or in the preparation stage which involves rehydration in water or in the buffered aqueous solution, typically stage (iii), during the hydration step of the lipid vesicles.

The cholesterol used in the preparation of liposomes has the function of stabilizing the lipid *bilayer* of the obtained liposomal nanovectors and modulating the chemical-physical parameters, more particularly the trapping efficiency, or quantity of hydrophilic, lipophilic, amphipathic, loaded drug in the liposomal formulation, and the kinetics of release, or quantity of drug released in the specific or target tissue, from the liposomal nanovectors object of the invention. Cholesterol can be added in quantities ranging from 0.1 to 3 in molar fraction with respect to phospholipids and phospholipid-PEG-MAL and / or phospholipid-PEG-KillerTRAIL.

For lipophilic drugs we indicate but not limited to: paclitaxel, docetaxel, campothecina, etoposide, anhydrous base doxorubicin, anhydrous base irinotecan, vascular endothelial growth factor (VEGFR) inhibitors (e.g. Cabozantinib, Nintedanib), Wnt / β-catenin modulators XAV-939 (CAS NO. 284028-89-3), ICG-001 (CAS NO.

780757-88-2)), Hedgehog inhibitors (e.g. SANT75 [73], HPI1 Antibody or HIC0-4F9 (https: // www.thermofisher.com/antibody/product/HPi1-Antibody-clone-HIC0-4F9-Monoclonal/MA5-16126), PI3K (Phosphoinositide 3-kinases) / (Proteinkinase B) Akt / (*mammalian target of rapamycin*) mTOR *modulators*, which act on different portions of the receptors and modulate their activity neutrally, positively and negatively with a different level of efficacy and include receptors agonists and antagonists as well as receptors with partial agonist activity, inverse agonists, orthosteric and allosteric modulators, such as e.g. Rapamycin, Buparlisib; amphiphilic drugs: natural peptides with a efficacy that favors the inhibition of tumor growth of less than 50% compared to the control (untreated models) and the free drug, or produces a percentage cell viability lower than 50% compared to the control (untreated models) and the free drug.

For amphiphilic drugs according to their molecular weight and chemical-physical parameters, we indicate but not limited to some natural peptides, such as ovine lactofericin, magainin 2, hCAP109–135 (comprising the C-terminaldomain of human CAP18) and BMAP-28 from bovinemyeloid cathelicidin, neural (N) -cadherins, His-Ala-Val (HAV) sequence, Ala-Val (HAV), cyclic peptide N-Ac-CHAVC-NH2 or ADH-1 (Exherin), Cyclic peptides containing the sequence His-Trp-Gly-Phe (HWGF), peptideRS-83277, myelopep-tide-2 (MP-2), tyroserleutide (YSL), Lactoferricin, Angiotensin (1–7), Growth inhibitorypeptide (GIP), p53-derivedapoptotic peptide, Cilengitide, ATN-161, ADH-1 (Exherin), WX-360 (uPA), WX-360-Nle (uPA), ABT-510 (Throspondin-1), MDP, Tyroserleutide, Bestatin, AFPep, Hunter-killerpeptide, P15, with potent antitumor activity [58,59].

For hydrophilic drugs we indicate but not limited to gemcitabine hydrochloride [60], doxorubicin hydrochloride [61], cisplatin [62], irinotecan hydrochloride [63], carboplatin [64], oxaliplatin [65], 5-fluorine uracil [66], microRNA [67], capecitabine hydrochloride [68], erlotinib [69] and / or combinations thereof.

The advantage of the preparation procedure exemplified in point C) is to cover with micellar aggregates consisting of the phospholipid-PEG-protein conjugate, more particularly DSPE-PEG-KillerTRAIL, the liposomal formulations containing drugs, already on the market or currently used in clinical, such as but not exclusively Doxyl®/ Caelyx® (doxorubicin hydrochloride), AmBisome® (amphotericin B), DaunoXome® (Daunorubicin citrate salt), Myocet® (doxorucine hydrochloride), Marqibo® (vincristine sulfate), Mepact® (mifamurtide), Lipo-Dox® (doxorubicin hydrochloride), Visudyne® (verteporfin), Onivyde® (irinotecan), Lipusu® (paclitaxel), Epaxal®, (inactivated hepatitis A virus, virosomal (RG-SB strain)), Inflexal V® [inactivated, virosomal influenza virus surface antigen (A / California / 7/2009 (H1N1) pdm09 – derived strain used (NYMC X-181))] Lipoplatin® (cisplatin), Onpattro®, INN-patisiran [79]. In this way, a selective action is obtained in patients suffering from pathologies, as per the list below, thanks to the exposure of the apoptotic programmed cell death receptors of TRAIL, more particularly KillerTRAIL, on the surface of commercial liposomes.

The preparation procedure exemplified in point C) allows the creation of Lipo-PEG-KillerTRAIL starting from liposomes already present on the market and / or approved for clinical use by the FDA and the EMA, eliminating the steps necessary for the preparation of liposomes, thus favoring the possibility of obtaining selective liposomes towards cells and tissues that present on their surface the apoptotic receptors of programmed cell death of TRAIL, more particularly KillerTRAIL, directly in the clinical therapy units starting from a patient diagnosis that foresees the use of liposomes containing chemotherapeutic agents already present on the market and listed above, personalizing the patient's therapy with therapeutic liposomes present on the market or approved for clinical use against cells and tissues that have on their surface the apoptotic receptors for programmed cell death of TRAIL, more particularly the KillerTRAIL.

The protein, TRAIL and more preferentially KillerTRAIL, can be preliminarily purified by dialysis in buffer solution at pH 7.4 with the addition of 0.05 mM of EDTA inside a dialysis membrane with a *cut-off* of 2,000 Da. this operation is necessary to eliminate the dithiothreitol (DTT) present in the solution in which it is stored for transport and before its use the same protein, TRAIL and more preferentially KillerTRAIL.

A further subsequent procedure consists in carrying out a treatment of the protein solution with only 8 mM DTT at room temperature for 1 hour in order to avoid the spontaneous formation of disulfide bridges that would lead to the formation of the protein in the form of a trimer. To maintain the optimal conditions for the production of the conjugate, the protein solution from the DTT was further purified by means of a desalting chromatographic column. Under these experimental conditions the KillerTRAIL protein is found in monomeric form.

The degree of purity of the protein, TRAIL and more preferentially KillerTRAIL, was analyzed by a detector in UV / Vis spectroscopy coupled to a high performance chromatographic system in reverse phase or RP-HPLC.

The basic preparation method used to make liposomal vesicles consists in the evaporation of the solvent on a thin layer and relative formation of a phospholipid film on the glass surface of a graduated flask. This preparation method is referred to in technical language and is known to expert scientists in the field as *Thin Layer Evaporation* or TLE. The preparation can be carried out by solubilizing one or more neutral phospholipids, for example selected from the group listed below in list 1, alone or in association with cholesterol or other cationic and / or anionic phospholipids, together with those conjugated with the chains of PEG, PEGylated phospholipids or PEG-phospholipids, such as those selected in list group 2, 3 or 4, in apolar, polar aprotic or polar protic solvents or mixtures of solvents.

The mixture is then dried by eliminating the solvent and subsequently rehydrating the lipid film obtained with double distilled water or aqueous solutions consisting of phosphate buffer or PBS, pH 7.4. The formation of the liposomes is obtained through a process of mechanical stirring, for example with a speed comprised between 10 and 3,000 rpm, preferably between 10 and 700 rpm, more preferably between 10 and 300 rpm.

A preferred method of preparing the *bilayer* liposome phospholipidis as follows. Neutral lipids and cholesterol are used, listed in list 1 at different molar fractions (varying between 1: 9 and 9: 1). The mixture of phospholipids used is solubilized in solvent or mixture of solvents as mentioned above, listed in the previous list. The solvent mixture is then removed, for example by means of a vacuum evaporator (for example pressure between 100 and 700 mBar) and heated with a thermostated bath programmed in the temperature range between 30 and 80°C, connected to a diaphragm vacuum pump programmed at a pressure between 100 and 700 mBar.

The removal of the residual traces of the mixture of solvents from the obtained lipid film can then be carried out *over night* using a dryer, programmed at a temperature range between 30 and 80°C and connected to a membrane vacuum pump programmed at an interval pressure between 100 and 700 mBar.

The lipid film thus obtained is subsequently hydrated with an aqueous solution, for a volume between 1 and 100 ml, and the lipid suspension thus obtained is subjected to a number of alternating cycles of continuous stirring, between 3 and 20, and heating in a thermostated water bath, at a temperature between 30 and 80°C, for a number of cycles between 3 and 20.

The final concentration of lipids obtained for the liposomes is between 1 and 13.8 mM.

The heating of the liposomal suspension during the cycles of mechanical stirring and thermostated water bath, according to the times, temperatures and cycles reported in detail above, takes place at a temperature slightly higher than the transition temperature, Tm, of the phospholipids used for preparing the lipid film and obtaining the liposomes object of the invention.

In particular, by transition temperature or Tm we mean a temperature, characteristic for each phospholipid, below which the *bilayer* obtainedis in a gel phase (rigid structure) and above which the *bilayer* obtainedis in a liquid-crystalline phase (more fluid structure).

Tm is an important parameter to characterize the obtained liposomal vectors under the chemical-physical profile and in particular it indicates the state of rigidity and / or fluidity of the phospholipid *bilayer* which is able to modify the encapsulation of hydrophilic, lipophilic and amphipathic, previously described, and their release kinetics.

Tm is measured using the differential scanning calorimetry (DSC) method. Depending on the type of phospholipid used to prepare the liposomal vectors, and in particular of the fatty acid chains present in its structure, the type of charge of the polar head, the molar fractions, and the use of single phospholipids or mixtures of phospholipids and / or cholesterol contained in lists 1 to 4 below, the Tm of the phospholipids that form the *lipid* bilayer of the liposomes has a value in the range between -10 and 75°C.

The preparation of the liposomal formulations by post-conjugation of the therapeutic protein belonging to the family of the pro-apoptotic protein TRAIL, more precisely KillerTRAIL, was performed by adding the KillerTRAIL protein, converted into monomeric form as widely described previously in the invention, in ratio 4 / 1 with the phospholipid-PEG-MAL, more precisely DSPE-PEG-MAL taking into account that the PEG-MAL, in the hydration phase, is preferentially arranged on the external surface of

the liposomal vesicles, with the maleimide group exposed on the external surface of liposomes, in concentrations equivalent to about half of the compound compared to the quantity used to prepare the formulations. Therefore, considering 50% of the DSPE-PEG-conjugated reactive maleimide groups present externally on the surface of the liposomal vesicles, the exact amount of protein to be added to the liposomal suspension was calculated.

The phospholipid functionalized with PEG being in relationship with the one or more phospholipids according to the quantity used was chosen in the mole fraction range between 0:10 and 10: 0.

The obtained liposomal suspension was incubated for 3 h at room temperature. The formulations were subsequently dialyzed with a cellulose membrane with a cut-off of 50 kDa (Spectra / Por, dialysis membrane tubing, Fiscer Scientific Italia, Rodano (MI), Italy) against PBS pH 7.4 containing 0.05 mM of EDTA for 24 h. The post-conjugated liposomal system was finally evaluated by dynamic light scattering or DLS (Tables 1 and 2) and RP-HPLC (Figure 10).

The progress of the reaction was monitored by taking an aliquot (25 µl) of post-conjugated liposomes with the therapeutic protein belonging to the pro-apoptotic family TRAIL at predetermined times, preferably from 1 minute to 1 hour, more specifically particular KillerTRAIL, in monomeric form, for chromatographic analysis.

In relation to the above, by buffer solution we mean PBS, phosphate buffer, at a pH between 5 and 8, preferably between 6.8 and 7.5, because a lower pH would irreversibly denature the protein, however, a higher pH would allow the binding of amino groups of proteins, more reactive than thiols at pH <8.

Below are the lists of usable phospholipids.

LIST I - Neutral phospholipids: cholesterol and cholesterol derivatives; L-α-phosphatidylcholine (Egg, Chicken); L-α-phosphatidylcholine, hydrogenated (Egg,

Chicken); L-α-phosphatidylcholine (95%) (Egg, Chicken); L-α-Phosphatidylcholine (Egg, Chicken-60%) (Total Egg Phosphatide Extract); L-α-lysophosphatidylcholine (Egg, Chicken); L-α-phosphatidylcholine (Soy); L-α-phosphatidylcholine, hydrogenated (Soy); L-α-phosphatidylcholine (95%) (Soy); L-α-Phosphatidylcholine (Soy-40%); L-α-Phosphatidylcholine, 20% (Soy); L-α-lysophosphatidylcholine (Soy); L-α-phosphatidylcholine (Heart, Bovine); L-α-phosphatidylcholine (Brain, Porcine); L-α-phosphatidylcholine (Liver, Bovine); 1,2-dilauroyl-sn-glycero-3-phosphocholine; 1,2-ditridecanoyl-sn-glycero-3-phosphocholine; 1,2-dimyristoyl-sn-glycero-3-phosphocholine; 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; 1,2-diphytanoyl-sn-glycero-3-phosphocholine; 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine; 1,2-distearoyl-sn-glycero-3-phosphocholine; 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine; 1,2-diarachidoyl-sn-glycero-3-phosphocholine; 1,2-dihenarachidoyl-sn-glycero-3-phosphocholine; 1,2-dibehenoyl-sn-glycero-3-phosphocholine; 1,2-ditricosanoyl-sn-glycero-3-phosphocholine; 1,2-dilignoceroyl-sn-glycero-3-phosphocholine; Cis- 1,2-dinervonoyl-sn-glycero-3-phosphocholine; 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine; 1-myristoyl-2-stearoyl-sn-glycero-3-phosphocholine; 1-palmitoyl-2-acetyl-sn-glycero-3-phosphocholine; 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine; 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine; 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; 1-stearoyl-2-palmitoyl-sn-glycero-3-phosphocholine; 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine; 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine; 1-oleoyl-2-myristoyl-sn-glycero-3-phosphocholine; 1,2-bis (10,12-tricosadiynoyl) -sn-glycero-3-phosphocholine; 1,2-bis (10,12-tricosadiynoyl) -sn-glycero-3-phosphoethanolamine; 1-palmitoyl-2- (10,12-tricosadiynoyl) -sn-glycero-3-phosphocholine; 1-palmitoyl-2- (10,12-

tricosadiynoyl) -sn-glycero-3-phosphoethanolamine (Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids).

LIST II - Cationic phospholipids: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt); 3ß- [N- (N', N'-dimethylaminoethane) -carbamoyl] cholesterol hydrochloride (DC-Cholesterol · HCl); 1,2-ditetradecanoyl-3-trimethylammonium-propane (chloride salt) (DMTAP); 1,2-dioleoyl-3-trimethylammonium-propane (methyl sulfate salt); 1,2-dihexadecanoyl-3-trimethylammonium-propane (chloride salt); 1,2-dioctadecanoyl-3-trimethylammonium-propane (chloride salt); 1-oleoyl-2- [6 - [(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] hexanoyl] -3-trimethylammonium propane (chloride salt); Fluorescent 1-oleoyl-2- [6 - [(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] hexanoyl] -3-trimethylammonium propane (chloride salt); N- (4-carboxybenzyl) -N, N-dimethyl-2,3-bis (oleoyloxy) propan-1-aminium (DOBAQ); 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); 1,2-dimyristoyl-3-dimethylammonium-propane; 1,2-dipalmitoyl-3-dimethylammonium-propane; 1,2-distearoyl-3-dimethylammonium-propane, dimethyldioctadecylammonium (bromide salt) (DDA); 1,2-dilauroyl-sn-glycero-3-ethylphosphocholine (chloride salt); 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (chloride salt); 1,2-dimyristoleoyl-sn-glycero-3-ethylphosphocholine (Tf salt); 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine (chloride salt); 1,2-distearoyl-sn-glycero-3-ethylphosphocholine (chloride salt); 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (chloride salt); 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (chloride salt); 1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA), N1- [2 - ((1S) -1 - [(3-aminopropyl) amino] -4- [di (3- amino-propyl) amino] butylcarboxamido) ethyl] -3,4-di [oleyloxy] -benzamide (Next Polar Lipids_https://avantilipids.com/product-category/polymers-polymerizable-lipids).

List III - Anionic phospholipids: L-α-phosphatidic acid (Egg, Chicken); L-α-phosphatidic acid (Soy) (sodium salt); 1,2-dihexanoyl-sn-glycero-3-phosphate (sodium salt); 1,2-

dioctanoyl-sn-glycero-3-phosphate (sodium salt); 1,2-didecanoyl-sn-glycero-3-phosphate (sodium salt); 1,2-dilauroyl-sn-glycero-3-phosphate (sodium salt); 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt); 1,2-dimyristoyl-sn-glycero-3-phosphate (sodium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphate (sodium salt); 1,2-diphytanoyl-sn-glycero-3-phosphate (sodium salt); 1,2-diheptadecanoyl-sn-glycero-3-phosphate (sodium salt); 1,2-distearoyl-sn-glycero-3-phosphate (sodium salt); 1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt); 1,2-dilinoleoyl-sn-glycero-3-phosphate (sodium salt); 1,2-dilinoleoyl-sn-glycero-3-phosphate (sodium salt); 1,2-diarachidonoyl-sn-glycero-3-phosphate (sodium salt); 1,2-didocosahexaenoyl-sn-glycero-3-phosphate (sodium salt); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt); 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphate (sodium salt); 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt); 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (sodium salt); 1-stearoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt); 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphate (sodium salt); 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt); 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (sodium salt); 1-hexanoyl-2-hydroxy-sn-glycero-3-phosphate (ammonium salt); 1-myristoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt); 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt); 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt); 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt); 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt); 1-linoleoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt); 1-arachidonoyl-2-hydroxy-sn-glycero-3-phosphate (ammonium salt); 1-O-hexadecyl-2-hydroxy-sn-glycero-3-phosphate (ammonium salt); 1-O-octadecyl-2-hydroxy-sn-glycero-3-phosphate (ammonium salt); 1- (9Z-octadecenyl) -2-hydroxy-sn-glycero-3-phosphate (ammonium salt); 1-palmitoyl-sn-glycero-2,3-cyclic-phosphate (ammonium salt); 1-heptadecanoyl-glycero-2,3-cyclic-phosphate (ammonium salt); 1-oleoyl-sn-glycero-2,3-cyclic-phosphate (ammonium salt); 1-O-

hexadecyl-sn-glycero-2,3-cyclic-phosphate (ammonium salt); 1-O- (9Z-octadecenyl) -sn-glycero-2,3-cyclic-phosphate (ammonium salt); 1-oleoyl-2-methyl-sn-glycero-3-phosphothionate (ammonium salt); 1-oleoyl-2-methyl-sn-glycero-3-phosphothionate (ammonium salt); (S) -phosphoric acid mono- {2-octadec-9-enoylamino-3- [4- (pyridin-2-ylmethoxy) -phenyl] -propyl} ester (ammonium salt); (S) -phosphoric acid mono- {2-octadec-9-enoylamino-3- [4- (pyridin-2-ylmethoxy) -phenyl] -propyl} ester (ammonium salt); (R) -phosphoric acid mono- {2-octadec-9-enoylamino-3- [4- (pyridin-2-ylmethoxy) -phenyl] -propyl} ester (ammonium salt); N-palmitoyl-serine phosphoric acid (ammonium salt); N-palmitoyl-tyrosine phosphoric acid (ammonium salt); L-α-phosphatidylglycerol (Egg, Chicken) (sodium salt); L-α-phosphatidylglycerol (Soy) (sodium salt); L-α-phosphatidylglycerol (E. coli) (sodium salt); 1,2-dihexanoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dioctanoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-didecanoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dilauroyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dimyristoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dipentadecanoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dipalmitoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-diphytanoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-diheptadecanoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-distearoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); cis-1,2-dioleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); trans-1,2-dielaidoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dilinoleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-dilinolenoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1,2-diarachidonoyl-sn-glycero-3- [phospho-rac- (1-glycerol)] (sodium salt); 1,2-didocosahexaenoyl-sn-glycero-3- [phospho-rac- (1-glycerol)] (sodium salt); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-palmitoyl-2-linoleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-palmitoyl-2-arachidonoyl-sn-

glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-stearoyl-2-oleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-myristoyl-2-hydroxy-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-stearoyl-2-hydroxy-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); 1-oleoyl-2-hydroxy-sn-glycero-3-phospho- (1'-rac-glycerol) (sodium salt); L-α-phosphatidylserine (Brain, Porcine) (sodium salt); L-α-lysophosphatidylserine (Brain, Porcine) (sodium salt); L-α-phosphatidylserine (Soy, 99%) (sodium salt); 1,2-dihexanoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-dioctanoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-didecanoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-dilauroyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-diphytanoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-distearoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-diarachidonoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1,2-didocosahexaenoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-palmitoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-

stearoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine (sodium salt); 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phospho-L-serine (sodium salt); L-α-lysophosphatidylserine (Brain, Porcine) (sodium salt); 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (sodium salt); 1- (10Z-heptadecenoyl) -2-hydroxy-sn-glycero-3-[phospho-L-serine] (sodium salt); 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (sodium salt); 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (sodium salt); L-α-phosphatidylinositol (Liver, Bovine) (sodium salt); L-α-lysophosphatidylinositol (Liver, Bovine) (sodium salt); L-α-phosphatidylinositol (Soy) (sodium salt); L-α-lysophosphatidylinositol (Soy) (sodium salt); L-α-phosphatidylinositol-4-phosphate (Brain, Porcine) (ammonium Salt); L-α-phosphatidylinositol-4,5-bisphosphate (Brain, Porcine) (ammonium salt); 1,2-dioctanoyl-sn-glycero-3-phospho- (1'-myo-inositol) (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phospho- (1'-myo-inositol) (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoinositol (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol) (ammonium salt); 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol (ammonium salt); 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoinositol (ammonium salt); 1,2-dioctanoyl-sn-glycero-3- (phosphoinositol-3-phosphate) (ammonium salt); 1,2-dioctanoyl-sn-glycero-3-phospho- (1'-myo-inositol-4'-phosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-3'-phosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-4'-phosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-5'-phosphate) (ammonium salt); 1,2-dihexanoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 5'-bisphosphate) (ammonium salt); 1,2-dioctanoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 4'-bisphosphate) (ammonium salt); 1,2-dioctanoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 5'-bisphosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-4', 5'-bisphosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 4'-bisphosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-

3-phospho- (1'-myo-inositol-3', 5'-bisphosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-4', 5'-bisphosphate) (ammonium salt); 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 5'-bisphosphate) (ammonium salt); 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho- (1'-myo-inositol-4', 5'-bisphosphate) (ammonium salt); 1,2-dihexanoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 4', 5'-trisphosphate) (ammonium salt); 1,2-dioctanoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 4', 5'-trisphosphate) (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 4', 5'-trisphosphate) (ammonium salt); 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho- (1'-myo-inositol-3', 4', 5'-trisphosphate) (ammonium salt); L-α-lysophosphatidylinositol (Liver, Bovine) (sodium salt); L-α-lysophosphatidylinositol (Soy) (sodium salt); 1-tridecanoyl-2-hydroxy-sn-glycero-3-phospho- (1'-myo-inositol) (ammonium salt); 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoinositol (ammonium salt); 1-stearoyl-2-hydroxy-sn-glycero-3-phosphoinositol (ammonium salt); 1- (10Z-heptadecenoyl) -2-hydroxy-sn-glycero-3-phospho- (1'-myo-inositol) (ammonium salt); 1-oleoyl-2-hydroxy-sn-glycero-3-phospho- (1'-myo-inositol) (ammonium salt); 1-arachidonoyl-2-hydroxy-sn-glycero-3-phosphoinositol (ammonium salt) (Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids).

LIST IV - Phospholipids conjugated to polyethylene glycol chains: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-

phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 750]}; N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 2000]}; N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 5000]}; 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [dibenzocyclooctyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [azido (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [succinyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [carboxy (polyethylene glycol) -2000]

(ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [maleimide (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [PDP (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [biotinyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [cyanur (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [folate (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [folate (polyethylene glycol) -5000] (ammonium salt); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [poly (ethylene glycol) 2000-N'-carboxyfluorescein] (ammonium salt) (Next Polar Lipids https://avantilipids.com/product-category/polymers-polymerizable-lipids).

The liposomal nanovectors obtained that have the therapeutic protein belonging to the pro-apoptotic family TRAIL conjugated on their surface, more particularly the KillerTRAIL, in monomeric form, of which the general name will be Lipo-PEG-KillerTRAIL, can be used for all those pathologies in which the presence of TRAIL family receptors on the surface of cells and tissues was evaluated and in particular a series of tumor, immune, angiogenic andpathologies bone *turnover* (due to tropism towards the osteoprotegerin OPG receptor). The pathologies that are mainly referred to are various types of solid and liquid tumors. Among the most studied we report pancreatic cancer, non-small cell lung cancer, cervical cancer, mesothelioma, rhabdomyosarcoma, gliomas, chronic lymphocytic leukemia.

The Lipo-PEG-KillerTRAIL products can be used parenterally, together with vehicles such as water, buffers, physiological solutions, 5% (w / v) glucose solutions, stabilizers (e.g. antioxidants), chelating agents (e.g. ethylenediamino tetracetic acid or

EDTA), and can be produced under sterile conditions. The term parenteral defines the type of administration by injection through the external integuments of the skin and / or blood vessels or directly into the circulation (for example the intradermal route, the subcutaneous route, the intramuscular route and the intravenous route).

The Lipo-PEG-KillerTRAIL products can advantageously be administered in useful therapeutic doses, increasing the efficacy of the therapy in terms of duration of the therapeutic effect (effect due to the reduction of renal *clearance*) and reduction of the formation of protein and immune complex aggregates, the latter formed by the union of an antigen with the respective antibody. Immune complexes, depositing themselves in the tissues, cause diseases characterized by inflammatory reactions in the skin, joints, kidneys, small vessels: the best known are scleroderma, serum sickness, some glomerulonephritis.

Furthermore, the Lipo-PEG-KillerTRAIL products allow, for passive and active *targeting* phenomena, where by passive *targeting* we mean the ability that the Lipo-PEG-KillerTRAILs have to accumulate inside the tumor tissue, thanks to their properties of long circulation and nanometric dimensions, generally lower than 200 nm, and the characteristics of the endothelium of the tumor tissue which is very loose, has numerous fenestrations larger than 200 nm, has a lymphatic pressure higher than 20 mmHg, and a pH lower than 4. Accumulation by passive *targeting* of Lipo-PEG-KillerTRAIL in the tumor tissue therefore takes place by exploiting the physical and anatomo-physiological phenomena previously reported.

On the contrary, active *targeting* means the ability that the KillerTRAIL bound on the surface of Lipo-PEG-KillerTRAIL, according to the method previously described within the invention, has to bind to the specific receptors of the family of pro-apoptotic proteins of TRAIL, more specifically, KillerTRAIL, expressed on the tumor cells and tissues described above, and to favor a specific accumulation of Lipo-PEG-KillerTRAIL

within the tumor tissue with the consequent decrease of the dose accumulated in healthy tissues and an increase in dose accumulated in the tumor tissue.

Thanks to these effects, therefore, the nanosystem is transferred from the blood flow to the tumor site and the derivative of the TRAIL family, more preferably KillerTRAIL, will select, within the tissue, the healthy cells from the diseased ones (over-expressing the death receptors) which therefore they will suffer more the pro-apoptotic effect induced by the cytokine.

This synergistic effect as a whole (active and passive) increases the therapeutic effect of the cytokine and potentially also of the drugs encapsulated within the nanosystem, minimizing the presence of chemotherapy in other parts of the body, with a significant decrease in side effects.

The cytokine KillerTRAIL, in monomeric form, belonging to the superfamily of *Tumor Necrosis Factor*, was covalently linked on the surface of the liposomal vectors with a particular innovative approach through the binding on cysteine residues of the protein in position 230 as already described previously in the invention. In fact, the KillerTRAIL protein, in monomeric form, conjugated on the surface of Lipo-PEG-KillerTRAIL, unlike the native protein or other recombinant proteins, is much more stable and its powerful biological activity is maintained despite the fact that the trimerization of the protein itself is prevented.

The greater activity of Lipo-PEG-KillerTRAIL compared to the native KillerTRAIL protein is confirmed by experiments on *in vivo* mouse models obtained on Balb / c mice implanted (*xenograft*) with HTC116 colon carcinoma cells (Example 8, Figure 10). In fact, the data obtained confirm the improvement of the pharmacokinetic and biopharmaceutical characteristics of KillerTRAIL compared to the native form (Example 8, Figure 9). The use of liposomal vectors of the Lipo-PEG-KillerTRAIL type therefore allows the improvement of the biopharmaceutical and pharmacokinetic potential of the

native protein (KillerTRAIL) (Example 8, Figure 9) and also allows the co-administration in a synergistic way with hydrophilic, lipophilic and amphipathic, or other amphipathic therapeutic peptides, such as those previously reported in the description of the invention, in order to reduce the drug resistance of some cell types having receptors but not responding to the therapeutic KillerTRAIL protein in monomeric form.

The increase in therapeutic activity on *in vivo* mouse models, obtained on Balb / c mice implanted (*xenograft*) with colon carcinoma cells HTC116, for Lipo-PEG-KillerTRAIL compared to free KillerTRAIL (Example 8, Figure 10), and the enhancement of its therapeutic activity, possibly also in co-administration synergistically with hydrophilic, lipophilic and amphipathic drugs, or other amphipathic therapeutic peptides, such as those previously reported in the description of the invention, is further highlighted by comparing the data obtained on the same models of Balb / c mice implanted (*xenograft*) with cells colon carcinoma HTC116 for the phospholipid-PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 13) and the phospholipid-PEG-KillerTRAIL, the PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 14).

Lipo-PEG-KillerTRAIL also significantly increases the pharmacokinetics of KillerTRAIL compared to free KillerTRAIL and the phospholipid derivative-PEG-KillerTRAIL (Example 8, Table 5). Furthermore, the presence of KillerTRAIL, in monometric form, conjugated by means of the residual Cys-230 to the PEG chains through a maleimmiidic bond, favors the specific accumulation of the therapeutic proietin within the tumor tissue, and neither reduces its distribution in the organs. of the reticuloendothelial system and in the other tissues of the organism in which the KillerTRAIL protein does not have a selective action (Example 8, Figure 15 AC).

Lipo-PEG-KillerTRAIL can therefore be proposed as a potential innovative system for anticancer treatment and can be enrolled in clinical trials for the alternative therapeutic treatment to conventional chemotherapy. It guarantees the significant

decrease in direct toxicity (hepatotoxicity) and indirect (formation of immune complexes, tumor resistance) and the potential reduction of the cell resistance phenomenon due to the synergistic activity of anticancer drugs included within them, without further burdening the patient's health.

The greatest benefit is therefore found in the reduction of convalescence problems after therapeutic treatment, longer times between one dose and the next, fewer side effects (mild or severe), a targeted and selective action against tumor cells and tissues that over express the receptors of the pro-apoptotic TRAIL family, more particularly KillerTRAIL, in monomeric form, on their surface, a reduction in the therapeutic dose effective for the treatment of the disease, an increase in the therapeutic efficacy of the drug delivered, the co-stimulation of the patient's immune system in the therapeutic response to drug treatment, an improvement in drug biodistribution, a reduction in systemic *clearance*, an increase in the circulatory half-life and systemic biodistribution, the possibility of co-vehiculating several drugs with different chemical-physical properties, the possibility of realizing a therapeutic treatment with multiple drugs instead of administering multiple drugs at different time intervals, an increase in patient *compliance*, a longer stay of the drug in the systemic circulation, a reduced metabolism and / or inactivation, and, last but not least, an increase in expectations of life of the patient.

Among the most studied pathologies we report pancreatic carcinoma (ductal adenocarcinoma of the pancreas and neuroendocrine tumors, which originate from the cells of the islets of *Langerhans*), non-small cell lung cancer (squamous cell carcinoma), large cell carcinoma and adenocarcinoma, which in turn is subdivided into sarcomatosis and lung lymphomas), cervical cancer (squamous cell carcinoma and adenocarcinoma), mesothelioma (which affects and differs in various tissues: lung (pleura), heart (pericardium), intestine (peritoneum) and testes (vaginal tunica), rhabdomyosarcoma

(embryonic and alveolar), gliomas and related subtypes (glioblastoma, anaplastic astrocytoma, diffuse astrocytoma, pilocytic astrocytoma, other astrocytomas, oligodendroglioma, other lymphocytoma and laukemia chronic.

Pharmaceutical preparations containing liposomes which have the pro-apoptotic protein TRAIL on their surface, more in p articulating the KillerTRAIL, in monomeric form, according to the invention can be obtained by adding and / or mixing the aforementioned liposomal vectors with adjuvants, excipients or stabilizers that meet the requirements for the excipients for pharmaceutical use present on the market [65], in the form of lyophilized formulations and aqueous suspensions. The vectors, excipients or stabilizers used are preferably selected from: buffers such as tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), 4- (2-hydroxyethyl) -1-piperazine-ethanesulfonic acid (HEPES), phosphate, citrate and other organic acids; antioxidants including ascorbic acid and methionine; preservatives, such as octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl or benzyl alcohol, alkyl parabens (such as, but not limited to, hydroxybenzoic acid, methyl parabens (E number E218), ethyl parabens (E214) (E216), butyl paraben, heptyl parabens (E209), isobutyl parabens, isopropyl parabens, benzyl parabens and their salts); catechol; resorcinol; cyclohexanol; 3-pentanol; low molecular weight polypeptides (such as compounds containing less than about 10 amino acid residues with a molecular weight in the range of 10 to 1,000 Da); proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose or dextrins; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter ions such as sodium; and / or non-ionic surfactants such as TWEEN™, PLURONICS™ or further polyethylene glycol (PEG).

Further examples of these excipients include ion exchangers, such as alumina, aluminum stearate; lecithin; buffer substances, such as glycine, sorbic acid, potassium sorbate; mixtures of partial glycerides of saturated vegetable fatty acids; water; salts, or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride; colloidal silica; magnesium trisilicate; polyvinyl-pyrrolidone and cellulose-based substances.

The excipients for making pharmaceutical forms for topical applications or gels include: polysaccharides, such as carboxy-methyl-cellulose or methyl-cellulose, polyvinylpyrrolidone, polyacrylates, ethylene-propylene block polymers, polyethylene glycol and waxes.

For all administrations, other modified release pharmaceutical forms are also suitably used. Such forms include, for example, patches, inhalation forms, nasal sprays, sublingual tablets and extended-release preparations.

The liposomal vectors which present on their surface the therapeutic protein TRAIL, more particularly KillerTRAIL, in the monomeric form, object of the invention, to be used for *in vivo* administration must be sterile and non-pyrogenic. This is easily accomplished by filtration through filtering and sterilizing membranes, before or after lyophilization and their reconstitution in the form of a suspension at the time of administration. The liposomal vectors which present on their surface the therapeutic protein TRAIL, more particularly KillerTRAIL, object of the invention, are normally preserved in anhydrous form, for example lyophilized if used for systemic administration. If in lyophilized form, they are also formulated in combination with other ingredients, belonging to those listed above, for reconstitution with an appropriate diluent at the time of use.

An example of a liquid formulation is a sterile, clear, colorless, unpreserved suspension filled in a single-dose vial for subcutaneous, intramuscular, or intravenous

injection, or a sterile lyophilized powder, stored in a single-dose bottle to be resuspended with an appropriate vehicle. sterile, for example, sterile water, sterile physiological solution (Sodium Chloride or NaCl 0.9% w / v) or sterile glucose solution (Glucose 5% w / v), at the time of subcutaneous, intramuscular, or intravenous administration.

The formulations can for example be placed in a container provided with a sterile access port, for example a bag or a vial of intravenous solution having a cap that can be pierced by a hypodermic injection needle. The formulations are preferably administered as injections or repeated infusions intravenously (iv), subcutaneously (sc), intramuscularly (im), or as aerosols suitable for intra-nasal or intra-pulmonary delivery.

The liposomal vectors that present on their surface the therapeutic protein TRAIL, more particularly KillerTRAIL, in the monomeric form, described and produced here, can be used parenterally, together with vehicles such as water, buffer solutions, physiological solution, stabilizers (for example antioxidants), chelating agents (for example EDTA), and can be produced under conditions of sterility and apyrogenicity. The term parenteral defines the type of administration by lesion through the external integuments and injection of the drug directly into the circulation (for example the intradermal route, the subcutaneous route, the intramuscular route and the intravenous route).

The liposomal vectors which present on their surface the therapeutic protein TRAIL, more particularly KillerTRAIL, in the monomeric form, object of the invention, can be administered at different therapeutic doses, increasing the efficacy of the therapy in terms of duration of the therapeutic effect (effect due to the reduction of *renal clearance*) and reduction of the formation of immunocomplexes (due to the steric hindrance of the polymers which prevents the aggregation of more protein molecules). The optimal dosages will in any case be identified by the attending physician according to the type of disease and the patient's condition.

Furthermore, the liposomal vectors that present on their surface the therapeutic protein TRAIL, more particularly KillerTRAIL, in the monomeric form, object of the invention, allow the accumulation of KillerTRAIL in the tumor tissue, by passive *targeting*, exploiting the phenomenon of *Enhanced Permeation and Retention* (EPR). This type of phenomenon reduces the dose of KillerTRAIL that could accumulate in healthy tissues causing side effects. The presence of the pro-apoptotic protein TRAIL, more particularly KillerTRAIL, in monomeric form, on the surface of the liposomal vectors, allows the accumulation of liposomal vesicles and KillerTRAIL in the tumor tissue also for active *targeting*, exploiting the *over*-expression of the TRAIL pro-apoptotic family, more specifically KillerTRAIL, on the surface of cells and tissues that are sensitive to the therapeutic protein. This type of process considerably increases the dose of liposomal vesicles and of KillerTRAIL that accumulates in the pathological tissues, consequently reducing the dose that accumulates in healthy tissues with a significant reduction in the therapeutically effective dose and an increase in patient *compliance*.

As regards the use of the invention for diagnostic purposes, it is possible to predict the same synthesis using the same TRAIL protein, radio-labeled with ^{125}I [66,67] or ^{188}Re /^{188}W [68] and / or by labeling the vesicles liposomal with radio-labeled lipids, such as hexadecyl cholesteryl ether tritiate (^{3}HCE, or Cholesterol hexadecyl ether [cholesteryl-1,2-3H (N)], TLC purity ≥97%, Perkin Elmer-Italia, Monza (MI), Italy), which is a derivative of labeled cholesterol able to integrate into the lipid *bilayer* of liposomal vectors and remain stably in the bilayer without being exchanged [61]; LysoPtdCho-^{14}C-DHA; lysoPtdCho-DHA radiolabelled with carbon 14 [1-^{14}C]; NE-^{14}C-DHANE-DHA radiolabelled with carbon 14 [1-^{14}C] commercially available from American Radiolabeled Chemicals Inc. (St-Louis, MO, USA) and stably inserted within the lipid *bilayer* of liposomal vectors. The radiolabelled liposomal vectors, which express on their surface the therapeutic protein TRAIL, more particularly KillerTRAIL, in monomeric form, can be

traced, after systemic administration, by means of the classic imaging techniques such as nuclear magnetic resonance associated with positron emission and computerized axial tomography associated with positron emission tomography (respectively MRI / PET and CT / PET).

Pharmaceutical preparations that contain the conjugates of the pro-apoptotic protein TRAIL, and in particular KillerTRAIL, in monomeric form, can also be administered in combination with other therapeutic agents additional to the formulation itself to have a synergistic effect, such as: 5-fluorouracil, irinotecan (Campothecin-11 or CPT-11) [69] and / or be administered in combination with ionizing radiation [70] and / or even radio-labeled diagnostic agents, such as ^{125}I, ^{188}Re, ^{188}W and relative combinations. It is important to highlight that the Apo-2L TRAIL protein, and consequently also the KillerTRAIL variant, are proteins that exist in a trimeric form and as such carry out their therapeutic action. The conjugation through a PEGylation process of TRAIL or KillerTRAIL, in monomeric form, on the surface of the liposomal vectors, according to the invention, has instead produced a new macromolecular entity containing the PEG polymer, at different molecular weights as indicated above, and a protein of the TRAIL family, in particular **KillerTRAIL, which remains monomeric even after administration.** This new macromolecular entity has been shown to improve the therapeutic and biopharmaceutical properties of liposomal vectors containing conjugated KillerTRAIL on their surface, compared to unconjugated trimeric TRAIL. The improvement is to be considered on the various points listed below:

1) The dosage used is significantly lower;
2) The therapeutic efficacy is maintained or even increased over time after the administration of PEGhylated liposomes containing on the surface the KillerTRAIL, in monomeric form;

3) The PEGhylated liposomal vectors that have KillerTRAIL on the surface, in monomeric form, are non-toxic after systemic administration in animal models used for experimentation.

For those pathologies for which the positive effects of this therapy have been ascertained, the advantages of the invention initially concern the increase in the plasma half-life of KillerTRAIL, compared to the native protein, due to its conjugation on the surface of the PEGhylated liposomal vesicles which are able to protect the protein from degradation by plasma enzymes. This leads to a temporal increase in therapeutic activity and therefore to a decrease in the doses administered, or to a prolongation of the times that elapse between one dose and the next (interval between doses administered).

The decrease in the concentration per single dose or the decrease in the number of doses necessary to obtain the therapeutic effect, reduces the toxic effect of KillerTRAIL (hepatotoxicity, toxicity at the injection site and serious side effects due to the increase in stimulation of the receptors of the TNF also in healthy tissues, such as MOF, *multiple organ failure*) and also increases patient *compliance*. All these positive effects improve the therapeutic approach of the previously listed disabling diseases (Table 1).

The conjugation of the KillerTRAIL protein to the PEGylated liposomal vectors, which present the PEG on their outer surface, was carried out directly on the cys 230, and unexpectedly the conjugation process resulted in an increase in the stability, biopharmaceutical properties and activity of the native protein. of the TRAIL family, in particular of the KillerTRAIL. As can clearly be seen from Figure 10, Example 8, the PEGylated liposomal vectors, which have the KillerTRAIL protein on the surface, maintain their therapeutic activity at very low doses compared to the control (mice treated with physiological solution (NaCl 0.9% w / v), and compared to KillerTRAIL, where the reduction in tumor volume is significant even 15 days after the end of treatment. The

conjugation of KillerTRAIL on the surface of PEGhylated liposomal vectors, however, increases the therapeutic efficacy of the KillerTRAIL protein by 60% compared to to KillerTRAIL (31.25%). The therapeutic protocol provides for the daily administration of 0.04 mg / kg (corresponding to the KillerTRAIL protein) for 7 days (therefore equivalent to 0.28 mg / kg of Lipo-PEG-KillerTRAIL equivalent to the amount of native protein) and the evaluation of the change in tumor volume from time 0 (first injection) to the next 15 days starting from the seventh day i treatment, for a total of 21 days. The experimental results obtained show that Lipo-PEG-KillerTRAIL increases:

1) The therapeutic efficacy of the native protein;
2) The therapeutic activity of the native protein after a single administration of Lipo-PEG-KillerTRAIL.

The therapeutic treatments on animal models, used forexperimentation *in vivo*, were stopped after 21 days of treatment as the animals, which develop the tumor, and used as a control, did not survive beyond 21 days of treatment, unlike those. treated with Lipo-PEG-KillerTRAIL.

Furthermore, the conjugation of the KillerTRAIL protein, in monomeric form, with the PEG present on the surface of the liposomal vectors, on the only cysteine residue present in the native protein in position 230 (cys 230), prevents the trimerization of the pro-apoptotic protein TRAIL, and in particular KillerTRAIL. This procedure therefore allows not only to PEGylate the single monomer of the KillerTRAIL protein, but also to prevent its trimerization.

Experimental data, relating to the MALDI-TOF mass spectroscopy study, carried out on the PEG2000 Da conjugate with the KillerTRAIL, indicate the presence of fragmentation peaks referred to the absolute molecular weight of PEG (2,119.4 Da) and of the KillerTRAIL PEGylated monomer (24,008.6 Da) (Example 9, Figure 11). In fact, the molecular weight of the single filament of KillerTRAIL corresponds to about 22,000

Da, as reported by the manufacturer, and as present in the reference database Expasy.org (https://www.expasy.org). This effect is highlighted in the activity and efficacy of Lipo-PEG-KillerTRAIL in an absolutely unexpected way, guaranteeing the further advantage of a co-administration of TRAIL with lipophilic or amphiphilic anticancer drugs co-conveyed using the PEGylated liposomal nano-vectors that present on their surface the KillerTRAIL (Example 8, Figure 10). The difference between Lipo-PEG-KillerTRAIL and KillerTRAIL was studied and, if it is necessary to co-administer lipophilic or amphipathic drugs, the data obtained were statistically significant (Example 8, Figure 10).

As an example, within the invention, the graph relating to the comparison of Lipo-PEG-KillerTRAIL and native KillerTRAIL for the reduction of the tumor mass volume in mice implanted (*xenographed*) with HTC116 colon carcinoma cells (Example 8, Figure 10). These data demonstrate that Lipo-PEG-KillerTRAIL has a statistically significant effect on the reduction of tumor mass both compared to the control and to KillerTRAIL.

The increase in therapeutic activity onmouse models *in vivo*, obtained on Balb / c mice implanted (*xenograft*) with colon carcinoma cells HTC116, for Lipo-PEG-KillerTRAIL compared to free KillerTRAIL (Example 8, Figure 10), and the enhancement of its therapeutic activity, possibly also in co-administration synergistically with hydrophilic, lipophilic and amphipathic drugs, or other amphipathic therapeutic peptides, such as those previously reported in the description of the invention, is further highlighted by comparing the data obtained on the same models of Balb / c mice implanted (*xenograft*) with cells colon carcinoma HTC116 for the phospholipid-PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 13) and the phospholipid-PEG-KillerTRAIL, the PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 14).

Furthermore, the presence of KillerTRAIL, in monometric form, conjugated by means of the residual Cys-230 to the PEG chains through a maleimmiidic bond, favors

the specific accumulation of the therapeutic proietin within the tumor tissue, and neither reduces its distribution in the organs. of the reticuloendothelial system and in the other tissues of the organism in which the KillerTRAIL protein does not have a selective action (Example 8, Figure 15 AC).

This reduction obtained on the volume of the tumor mass could be further improved by co-conveying within the liposomal formulation a hydrophilic, lipophilic or amphipathic drug, which could have a synergistic action with the native protein, reducing its resistance.

The increase in thetherapeutic efficacy *in vivo* of Lipo-PEG-KillerTRAIL is an unexpected result, as the steric hindrance of the PEG could have prevented the binding of the native KillerTRAIL protein with the programmed cell death receptors, expressed on the surface of the membranes of the cells, and thus reduce their therapeutic effect.

For lipophilic drugs, to be co-conveyed within the *lipid* bilayer of Lipo-PEG-KillerTRAIL, we indicate but not limited to: paclitaxel, docetaxel, campothecina, etoposide, doxorubicin anhydrous base, irinotecan anhydrous base, vascular endothelial growth factor (VEGFR) inhibitors (eg. Cabozantinib, Nintedanib), Wnt / β-catenin modulators (eg. XAV-939 (CAS NO. 284028-89-3), ICG-001 (CAS NO. 780757-88-2), Hedgehog inhibitors (eg. SANT75 [73], HPI1 Antibody or HIC0-4F9 (https://www.thermofisher.com/antibody/product/HPi1-Antibody-clone-HIC0-4F9-Monoclonal/MA5-16126), PI3K (Phosphoinositide 3-kinases) / (Protein kinase B) Akt / (mammalian target of rapamycin) mTOR modulators (eg Rapamycin, Buparlisib) and / or their combinations.

For amphipathic drugs, to be co-vehiculated within the *lipid* bilayer of Lipo-PEG-KillerTRAIL, according to their molecular weight and chemical-physical parameters, we indicate but not limited to some natural peptides, such as ovine lactofericin, magainin 2, hCAP 109–135 (comprising the C-terminaldomain of human CAP18) and BMAP-28 from

bovinemyeloid cathelicidin, neural (N) -cadherins, His-Ala-Val (HAV) sequence, Ala-Val (HAV), cyclic peptide N-Ac -CHAVC-NH2 or ADH-1 (Exherin), Cyclic peptides containing the sequence His-Trp-Gly-Phe (HWGF), peptide RS-83277, myelopep-tide-2 (MP-2), tyroserleutide (YSL), Lactoferricin, Angiotensin (1–7), Growth inhibitorypeptide (GIP), p53-derivedapoptotic peptide, Cilengitide, ATN-161, ADH-1 (Exherin), WX-360 (uPA), WX-360-Nle (uPA), ABT- 510 (Throspondin-1), MDP, Tyroserleutide, Bestatin, AFPep, Hunter-killerpeptide, P15, with potent antitumor activity [58,59] and / or combinations thereof.

For hydrophilic drugs, to be co-vehiculated within the aqueous core of Lipo-PEG-KillerTRAIL, we indicate but not limited to: gemcitabine hydrochloride [60], doxorubicin hydrochloride [61], cisplatin [62], irinotecan hydrochloride [63], carboplatin [64], oxaliplatin [65], 5-fluoro uracil [66], microRNA [67], capecitabine hydrochloride [68], erlotinib [69] and / or combinations thereof.

This approach has made it possible to obtain a stable formulation, which is able to overcome the problems related to the stability, aggregation and low half-life of the native protein and its natural predisposition to aggregate in an aqueous environment thus forming trimers, and thus obtaining an improvement in pharmacokinetic parameters. of biodistribution and a better antitumor and immunostimulating activity of the native protein.

The presence of the protein on the surface of Lipo-PEG-KillerTRAIL promotes their targeting capacity towards tumor cells that express on their surface the receptors of the TRAIL family. All the PEG conjugated with phospholipid and the PEG conjugated with phospholipid and functionalized with maleimide, used to prepare the Lipo-PEG-KillerTRAIL, object of the invention, and reported above, are commercial products distributed by Avanti Polar Lipids (https: / /avantilipids.com), its European distributor Sigma-Aldrich (https://www.sigmaaldrich.com), NanoCS (http://www.nanocs.com),

Creative PEGWORKS (https://www.creativepegworks.com), BioCHemPEG (https://www.biochempeg.com/content/xtheme/hunanhuateng/default.aspx), Iris Biotech (https://www.iris-biotech.de).

PEG plays a fundamental role in this structure, which acts as a copolymer covering the lipid *bilayer* liposome, and exposes the therapeutic protein on the surface of Lipo-PEG-KillerTRAIL without modifying its intrinsic activity towards target cells and tissues. which express on their surface the receptors of the TRAIL family. Furthermore, the PEG stabilizes the Lipo-PEG-KillerTRAIL obtained by preventing the formation of sulphide bridges and the consequent aggregation between the monomers of the protein. The amphipathic structure of the PEG also favors the adsorption on the surface of Lipo-PEG-KillerTRAIL of water-soluble molecules that can be co-conveyed with the water-soluble, liposoluble and amphipathic compounds encapsulated / complexed in the structure of the Lipo-PEG-KillerTRAIL.

Eventually, the *step* lyophilizationcan be preceded, followed or replaced by a *step* in which the Lipo-PEG-KillerTRAIL is combined with one or more hydrophilic, lipophilic and amphipathic drugs. It has been verified that this synthesis process does not modify the therapeutic activity of the native protein either *in vitro* or *in vivo* (Example 6, Figure 4).

Lipo-PEG-KillerTRAIL can be exploited to synergistically convey hydrophilic, lipophilic and amphipathic drugs, while maintaining both the targeting and the therapeutic activity of TRAIL, more particularly of KillerTRAIL, unaltered. In fact, hydrophilic drugs can be conveyed in the aqueous core or in the interlamellar aqueous compartments of Lipo-PEG-KillerTRAIL. For hydrophilic drugs we indicate but not limited to: gemcitabine hydrochloride [60], doxorubicin hydrochloride [61], cisplatin [62], irinotecan hydrochloride [63], carboplatin [64], oxaliplatin [65], 5-fluorine uracil [66], microRNA [67], capecitabine hydrochloride [68], erlotinib [69] and / or combinations thereof.

Lipophilic drugs can instead be conveyed / complexed in the double layer or *lipid* bilayer of Lipo-PEG-KillerTRAIL. For hydrophilic drugs we indicate but not limited to: paclitaxel, docetaxel, campothecina, etoposide, doxorubicin anhydrous base, irinotecan anhydrous base, *vascular endothelial growth factor* (VEGFR) inhibitors (e.g. Cabozantinib, Nintedanib), Wnt / β-catenin modulators -939 (CAS NO. 284028-89-3), ICG-001 (CAS NO. 780757-88-2), hedgehog inhibitors (e.g. SANT75 [70], HPI1 *Antibody* or HIC0-4F9 (https: // www. thermofisher.com/antibody/product/HPi1-Antibody-clone-HIC0-4F9-Monoclonal/MA5-16126)), PI3K (*Phosphoinositide 3-kinases*) / (*Protein kinase B*), Akt / (*mammalian target of rapamycin*), mTOR modulators (eg Rapamycin, Buparlisib) and / or their combinations.

Amphipathic drugs can be conveyed / complexed with their hydrophilic portion in the central aqueous compartment and / or in the interlamellar aqueous compartments, and with their lipophilic portion in the bilayer or bilayer. lipid content of Lipo-PEG-KillerTRAIL. For amphipathic drugs according to their molecular weight and chemical parameters physical, we indicate but not limited to some natural peptides, such as ovine lactofericin, magainin 2, hCAP109–135 (comprising the C-terminaldomain of human CAP18) and BMAP-28 from bovinemyeloid cathelicidin, neural (N) -cadherins, His-Ala -Val (HAV) sequence, Ala-Val (HAV), cyclic peptide N-Ac-CHAVC-NH2 or ADH-1 (Exherin), Cyclic peptides containing the sequence His-Trp-Gly-Phe (HWGF), peptideRS-83277, myelopep-tide-2 (MP-2), tyroserleutide (YSL), Lactoferricin, Angiotensin (1–7), Growth inhibitorypeptide (GIP), p53-derivedapoptotic peptide, Cilengitide, ATN-161, ADH-1 (Exherin), WX-360 (uPA), WX-360-Nle (uPA), ABT-510 (Throspondin-1), MDP, Tyroserleutide, Bestatin, AFPep, Hunter-killerpeptide, P15, with potent anticancer activity [58,59] and / or their combinations.

The PEG chains and the TRAIL protein cover the external surface of the Lipo-PEG-KillerTRAIL and arrange themselves towards the external aqueous environment

forming its polymeric coating. This spatial organization ensures that the native TRAIL protein, which has a targeting as well as a therapeutic action, is present on the surface of Lipo-PEG-KillerTRAIL and binds to tumor cells and tissues that express on their surface the receptors of the family of the BETWEEN.

The Lipo-PEG-KillerTRAILs have been characterized both from a chemical-physical and biological point of view.

Considering in particular the characteristics of KillerTRAIL [20], the delivery strategies adopted were such as to guarantee a modulation of the biopharmaceutical characteristics of the protein without altering the binding capacity with the cell substrate. The conjugation carried out on the thiol group of cysteine in the polypeptide chain of the protein, in particular of KillerTRAIL, did not affect the binding properties with the TRAIL receptor family.

Particularly preferred for the realization of Lipo-PEG-KillerTRAIL is the phospholipid-PEG compound, for example the one with a molecular weight of 2,000 Da, derivatized at the carboxyl end with a commonly used and absolutely non-toxic phospholipid, such as distearoyl-phosphatidyl -ethanolamine (DSPE) already present on the market and commonly used for the preparation of vesicular systems for pharmaceutical use [71]. This lipid, like other lipids, is included in the list of PEG conjugated phospholipids reported previously in the invention.

At the other end of the PEG polymer chain, functional groups of various types are generally present. For the purposes of the present invention it is preferable to use the maleimide group, which easily reacts with the thiol group of the protein, preferably KillerTRAIL, favoring the formation of a stable bond which acts as a bridge between the PEG and the protein. The presence of a maleimide group at the end of the PEG chain, opposite to that involved in binding with the phospholipid, thus allows binding with the cysteine in position 230 of the TRAIL protein chain, in particular KillerTRAIL, as

previously reported in the course of the invention, through the Michael symmetric addition reaction [57].

It has been verified that the compound thus obtained does not preclude the activity of KillerTRAIL, on the contrary, it reduces its short- and long-term toxicity and, remaining within the therapeutic window, increases the half-life of the protein itself, extending the therapeutic window. that is the time that elapses between one administration and another, increasing the molecular *targeting* on the cell substrate. Furthermore, it is important to underline that the proposed synthesis method is absolutely risk-free as no organic solvents or toxic compounds of any kind are used in any phase of the development of the synthesis scheme (Figure 7). This significantly increases the tolerability of the compound, with a low cost of the synthetic process and the speed in the purification steps of the conjugates from the unreacted molecules.

The synthesis scheme of the phospholipid-PEG-KillerTRAIL product used to obtain the lipid derivative to be inserted in the lipid *bilayer* of the liposomal vectors and subsequently to form the Lipo-PEG-KillerTRAIL object of the invention can therefore be summarized in its *steps* fundamental:

- Insert the phospholipid-PEG-active group in the lipid mixture used for the preparation of the liposomal vectors;
- Let the TRAIL protein or its derivative or its mutant, in particular KillerTRAIL, react with the phospholipid-PEG-group active in phosphate buffer for about 7 h at room temperature;
- Purify the compound thus obtained and, if necessary, proceed with further purification processes, from a minimum of 2 to a maximum of 10.

The process of the invention, which includes all the proteins of the TNF ligand family and in particular of TRAIL (also known as TNFSF10, TL2; APO2L; CD253; Apo-2L), of its mutant variants that differ in at least one position of amino

acids from the *wild type* TRAIL protein (also known as TNFSF10, TL2; APO2L; CD253; Apo-2L), (Entrez GeneID: 8743; access number NM_003810.2; UniProtKB / Swiss-Prot: P50591; UniProtKB / TrEMBL: Q6IBA9), of KillerTRAIL, in monomeric form, includes the following phases:

a. The protein was preliminarily purified by dialysis in phosphate buffer (PBS) at pH 7.4 in the presence of EDTA 0.05 mM, using dialysis membranes with a *cut-off* of 2,000 Da to eliminate the dithiothreitol (DTT) used in the storage solution of the protein itself. The purified sample was subsequently analyzed by UV / Vis spectroscopy and *Reverse Phase High Performance Liquid Chromatography* (RP-HPLC). **Under these experimental conditions the KillerTRAIL protein is found in monomeric form** after a modification process of the commercial product according to the procedure described in the invention.

b. The phospholipid-PEG-MAL and / or liposomal vectors presenting the phospholipid-PEG-maleimide exposed on their surface are added to the buffer solution containing the cytokine of the chosen TRAIL family, preferably KillerTRAIL (1 mg / mL), in monomeric form. molar ratio 1: 4 (protein: Lipo-PEG-MAL). The reaction mixture is kept under constant stirring at room temperature until the reaction is complete. The progress of the reaction can be monitored by taking an aliquot (25 µL) of sample at different incubation times for chromatographic analysis.

c. Once the reaction is complete, monitored by RP-HPLC (Example 5, Figure 8), the reaction product is purified again using a dialysis membrane (*cut-off* 30,000 Da) to eliminate the residual amount of protein that did not react during synthesis. of the compound.

d. The protein functionalized with the phospholipid-PEG present on the surface of the liposomal vectors can be lyophilized and can be stored in the temperature range from 0 to -32°C, more preferably, at the temperature of -20°C for subsequent uses.

e. The conjugate thus produced and purified can be administered as it is or, in the case in which it is chosen to co-administer in a synergistic way other hydrophilic, lipophilic or amphipathic drugs, the drug will be encapsulated / complexed in the aqueous core, in the aqueous interlamellar spaces and / or in the lipid *bilayer* as described above in the invention.

In relation to the previous points, by buffer solution we mean PBS, phosphate buffer, at a pH between 5 and 8, preferably between 6.8 and 7.5, because a lower pH would irreversibly denature the protein, however, a higher pH would allow the binding of amino groups of proteins, more reactive than thiols at pH <8.

Theexperimentation *in vivo* (Example 8, Figure 9, Table 5 and Figure 10) was performed on male rats (6 weeks of age, weight about 200 g) for the evaluation of the pharmacokinetics, and on Balb / c mice (4- 5 weeks of age, average weight 20-25 g), in which the tumor was developed (*xenograft*) by inoculation of HTC116 colon carcinoma cells.

The results obtained demonstrated an improvement in the pharmacokinetic parameters of Lipo-PEG-KillerTRAIL compared to KillerTRAIL alone (half-life or $t_{1/2}$ 28.34 times higher than Lipo-PEG-KillerTRAIL compared to the native protein KillerTRAIL (Example 8, Figure 9 and Table 5); iii) an increase in antitumor *in vivo* activity (Example 8, Figure 10).

Theantitumor *in vivo* activity on Balb / c mice, in whichwere implanted within the organ (*xenograft)* HTC116 colon carcinoma cells, was assessed by measuring the increase in tumor volume over a time span of 20 days from the start of therapy after administration of a dose equivalent to 0.04 mg / kg of free protein or Lipo-PEG-KillerTRAIL every 4 days (Example 8, Figure 10).

Lipo-PEG-KillerTRAIL proved to be more active than free KillerTRAIL, reducing tumor growth by 46% more than native KillerTRAIL (Example 8, Figure 10). The

differences obtained depend on the selective binding of Lipo-PEG-KillerTRAIL towards the tumor tissue, compared to the native protein KillerTRAIL, on its greater accumulation in the tumor mass and on the better biopharmaceutical and pharmacokinetic properties of the micellar derivative compared to the native protein (Example 8, Figure 9, Table 5 and Figure 10).

The increase in therapeutic activity onmouse models *in vivo*, obtained on Balb / c mice implanted (*xenograft*) with colon carcinoma cells HTC116, for Lipo-PEG-KillerTRAIL compared to free KillerTRAIL (Example 8, Figure 10), and the enhancement of its therapeutic activity, possibly also in co-administration synergistically with hydrophilic, lipophilic and amphipathic drugs, or other amphipathic therapeutic peptides, such as those previously reported in the description of the invention, is further highlighted by comparing the data obtained on the same models of Balb / c mice implanted (*xenograft*) with cells colon carcinoma HTC116 for the phospholipid-PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 13) and the phospholipid-PEG-KillerTRAIL, the PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 14).

Lipo-PEG-KillerTRAIL also significantly increases the pharmacokinetics of KillerTRAIL compared to free KillerTRAIL and the phospholipid derivative-PEG-KillerTRAIL (Example 8, Table 5).

Furthermore, the presence of KillerTRAIL, in monometric form, conjugated through the resiguous Cys-230 to the PEG chains through a maleimmiidic bond, favors the specific accumulation of the therapeutic proietin within the tumor tissue, and neither reduces its distribution in the organs. of the reticuloendothelial systems and in the other tissues of the organism in which the KillerTRAIL protein does not have a selective action (Example 8, Figure 15 AC). A further aim of this research was to evaluate in detail the targeting action of TRAIL for obtain a diagnostic effect, as well as therapeutic, in the presence of a radiolabelling agent inserted in Lipo-PEG-KillerTRAIL. TRAIL, in fact, has

active *targeting* properties that promote the binding of Lipo-PEG-KillerTRAIL towards the cells that express the receptors of the TRAIL family, in particular KillerTRAIL, thus also favoring the identification of vessels and / or tissues neoformates or metastases in the initial phase and also carrying out its powerful therapeutic activity there. The presence of these biopharmaceutical properties and the addition of the labeled radium make Lipo-PEG-KillerTRAIL a potential theranostic agent, as reported below.

The obtained Lipo-PEG-KillerTRAIL can be used for all those pathologies in which the presence of programmed cell death receptors (Apoptosis) of the KillerTRAIL family and related derivatives has been evaluated, implicated in a series of tumor and immune pathologies, angiogenetics and bone *turnover*.

TRAIL can bind to five different receptors found on a variety of cell types: four membrane receptors and one soluble receptor. Two of these membrane receptors, TRAIL-R1 / death receptor 4 (DR4) and TRAIL-R2 / death receptor 5 (DR5), act as agonist receptors, containing a cytoplasmic death domain through which TRAIL can transmit a signal apoptotic. The other two membrane receptors, TRAIL-R3 /*decoy receptor* 1 (DcR1) and TRAIL-R4 /*decoy receptor* 2 (DcR2), act as antagonist / regulatory receptors, lacking the domain responsible for programmed cell death.

In addition to these four transmembrane receptors, a soluble fifth receptor antagonist has been identified, osteoprotegerin (OPG), responsible for bone *turnover*. The pathologies that are mainly referred to are various types of solid and liquid tumors, inflammatory and autoimmune diseases that express apoptotic receptors of the TRAIL family, in particular KillerTRAIL, on the surface of cells and tissues (Table 1).

Table 1. Some pathologies whose cells over express programmed cell death receptors belonging to the cytokine TRAIL, in particular KillerTRAIL.

LIST OF PATHOLOGIES
Leukemia; Breast cancer Prostate cancer Lung cancer Renal cell carcinoma; Pancreatic cancer; Alzheimer's; Melanoma; Myelodysplastic syndrome; Lymphomas; Liver cancer; Randomyosarcoma; Glioma; Non-small cell lung cancer Hepatitis B virus; Hepatitis C virus; Fatty liver not related to alcohol abuse; Hepatic fibrosis; Cirrhosis; Mesothelioma; Pulmonary arterial hypertension; Systemic sclerosis; Cancer of the uterine cervix; Pulmonary fibrosis; Asthma; Flu virus; Atherosclerosis; Lupus erythematosus; Multiple sclerosis; HIV; Amyotrophic lateral sclerosis; Cytomegalovirus; Rheumatoid arthritis; Myasthenia gravis

Among the most studied pathologies we report pancreatic carcinoma (ductal adenocarcinoma of the pancreas and neuroendocrine tumors, which originate from the cells of the islets of *Langerhans*), non-small cell lung cancer (squamous cell carcinoma), large cell carcinoma and adenocarcinoma, which in turn is subdivided into sarcomatosis and lung lymphomas), cervical cancer (squamous cell carcinoma and adenocarcinoma), mesothelioma (which affects and differs in various tissues: lung (pleura), heart (pericardium), intestines (peritoneum) and testes (vaginal tunica), rhabdomyosarcoma (embryonic and alveolar), gliomas and related subtypes (glioblastoma, anaplastic astrocytoma, diffuse astrocytoma, pilocytic astrocytoma, other astrocytomas, oligodendroglioma and others), chronic lymphocytic leukemia.

Pharmaceutical preparations containing Lipo-PEG-KillerTRAIL according to the invention can be obtained by inserting in the pr liposomal preparations commercially available pharmaceutical adjuvants, excipients or stabilizers that meet the requirements for the excipients to be used [72], in the form of lyophilized formulations or aqueous suspensions.

The compounds, excipients or stabilizers used are preferably selected from: buffers such as tris- (hydroxymethyl) -aminomethane hydrochloride (Tris-HCl), 4- (2-

hydroxyethyl) -1-piperazine-ethanesulfonic acid (HEPES), phosphate, citrate and other organic acids; antioxidants including ascorbic acid and methionine; preservatives, such as octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl or benzyl alcohol, alkyl parabens (such as, but not limited to, hydroxybenzoic acid, methyl parabens (E number E218), ethyl parabens (E214) (E216), butylparaben, heptylparabens (E209), isobutylparabens, isopropylparabens, benzylparabens and their salts); catechol; resorcinol; cyclohexanol; 3-pentanol; low molecular weight polypeptides (such as compounds containing less than about 10 amino acid residues with a molecular weight in the range of 10 to 1,000 Da); proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose or dextrins; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter ions such as sodium; and / or non-ionic surfactants such as TWEEN™, PLURONICS™ or derivatives of polyethylene glycol (PEG).

Further examples of these excipients include ion exchangers, such as alumina, aluminum stearate; lecithin; buffer substances, such as glycine, sorbic acid, potassium sorbate; mixtures of partial glycerides of saturated vegetable fatty acids; water; salts, or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride; colloidal silica; magnesium trisilicate; polyvinyl-pyrrolidone and cellulose-based substances.

The excipients for making pharmaceutical forms for topical applications or gels include: polysaccharides, such as carboxy-methyl-cellulose or methyl-cellulose, polyvinylpyrrolidone, polyacrylates, ethylene-propylene block polymers, polyethylene glycol and waxes.

Lipo-PEG-KillerTRAILs to be used for *in vivo* administration must be sterile and non-pyrogenic. This is easily accomplished by filtration through sterilizing filtration membranes, before or after lyophilization and their reconstitution in the form of a suspension.

Lipo-PEG-KillerTRAIL are normally stored in anhydrous form, for example lyophilized, or in suspension if used for systemic administration. If in lyophilized form, Lipo-PEG-KillerTRAIL are typically formulated in combination with other ingredients, belonging to those listed above, for reconstitution with an appropriate diluent at the time of use. An example of a liquid formulation is a sterile, colorless, unpreserved suspension filled in a single-dose vial for subcutaneous, intramuscular, or intravenous injection.

The formulations can for example be placed in a container provided with a sterile access port, for example a bag or a vial of intravenous solution having a cap that can be pierced by a hypodermic injection needle. The formulations are preferably administered as injections or repeated infusions intravenously (iv), subcutaneously (sc), intramuscularly (im), or as aerosols suitable for intra-nasal or intra-pulmonary delivery.

Lipo-PEG-KillerTRAIL can be used parenterally, together with vehicles such as water, buffers, physiological solution, stabilizers (e.g. antioxidants), chelating agents (e.g. EDTA), and can be produced under sterile and apyrogenic conditions. The term parenteral defines the type of administration by lesion through the external integuments and injection of the drug directly into the circulation (for example the intradermal route, the subcutaneous route, the intramuscular route and the intravenous route).

The synthesized Lipo-PEG-KillerTRAIL can be administered at different therapeutic doses, increasing the efficacy of the therapy in terms of duration of the therapeutic effect (effect due to the reduction of renal *clearance*) and reduction of the formation of immune complexes (due to steric hindrance). of polymers which prevents the aggregation of multiple protein molecules). The optimal dosages will in any case be

identified by the attending physician according to the type of disease and the patient's condition.

Furthermore, the Lipo-PEG-KillerTRAILs allow the accumulation of KillerTRAIL in the tumor tissue, through passive *targeting*, exploiting the phenomenon of *Enhanced Permeation and Retention* (EPR). This type of phenomenon reduces the dose of KillerTRAIL that could accumulate in healthy tissues causing side effects.

As regards the use of the invention for diagnostic purposes, it is possible to predict the same synthesis using the same TRAIL protein, radio-labeled with ^{125}I [73,74] or ^{188}Re /^{188}W [75]. In addition to the substances listed above, the radio-labeled PEGhylated protein is added to the formulation to be administered in order to be able to trace it, after systemic administration, through classical imaging techniques such as, for example, nuclear magnetic resonance imaging associated with positron emission and computerized axial tomography associated with positron emission tomography (respectively MRI / PET and CT / PET). Also according to what has been reported in the literature [73], the presence of the radioisotope can increase the stability in plasma and the therapeutic efficacy, suggesting that radio-labeled Lipo-PEG-TRAIL:

- It reduces the side effects of the native protein belonging to the TRAIL family, in particular KillerTRAIL and increases the stability of the aforementioned native protein by reducing the potential formation of aggregates;
- It reduces the metabolism of the native protein and increases the diagnostic and therapeutic efficacy of the native protein belonging to the TRAIL family, and in particular KillerTRAIL.

Pharmaceutical preparations containing Lipo-PEG-KillerTRAIL can also be administered in combination with other therapeutic agents additional to the formulation itself to have a synergistic effect, such as: 5-fluorouracil, irinotecan (Campothecin-11 or CPT-11) [76] and / or be administered in combination with ionizing radiations [77] and /

or even radio-labeled diagnostic agents, such as ^{125}I, ^{188}Re, ^{188}W and relative combinations.

It is important to highlight that, according to the literature reported in the previous paragraphs, the Apo-2L TRAIL protein and consequently also the KillerTRAIL variant, are proteins that exist in a trimeric form and as such carry out their therapeutic action. Lipo-PEG-KillerTRAIL according to the invention has instead produced a new macromolecular entity containing the PEG polymer, at different molecular weights as indicated above, and a protein of the TRAIL family, in particular KillerTRAIL, **which remains monomeric even after administration**.

Lipo-PEG-KillerTRAILs have been shown to improve the therapeutic and biopharmaceutical properties of unconjugated trimeric TRAIL. The improvement is to be considered on the various points listed below:

1) The dosage used is significantly lower;
2) The therapeutic efficacy is maintained or even increased over time after the administration of Lipo-PEG-KillerTRAIL as well as phospholipid-PEG-KillerTRAIL;
3) Lipo-PEG-KillerTRAIL in the same way as PEG-KillerTRAIL and phospholipid-PEG-KillerTRAIL are non-toxic after systemic administration in animal models used for experimentation.

For those pathologies for which the positive effects of this therapy have been ascertained, the advantages of the invention initially concern the increase in the plasma half-life of KillerTRAIL, compared to the native protein, due to the presence of Lipo-PEG-KillerTRAIL, as well as of the mPEG or phospholipid-PEG polymer that manages to protect the protein from degradation due to plasma enzymes. This leads to a temporal increase in therapeutic activity and therefore to a decrease in the doses administered, or to a prolongation of the times that elapse between one dose and the next (interval between doses administered).

The decrease in the concentration per single dose or the decrease in the number of doses necessary to obtain the therapeutic effect, reduces the toxic effect of KillerTRAIL (hepatotoxicity, toxicity at the injection site and serious side effects due to the increase in stimulation of the receptors of the TNF also in healthy tissues, such as MOF, *multiple organ failure*) and also increases patient *compliance*. All these positive effects improve the therapeutic approach of the previously listed disabling diseases (Table 1).

PEGhylation of the KillerTRAIL protein was carried out directly on Cys 230, and unexpectedly the conjugation process resulted in an increase in the stability, biopharmaceutical properties and activity of the native protein of the TRAIL family, in particular of KillerTRAIL. As can clearly be seen from Figure 10 (Example 8), the PEGhylated protein maintains its therapeutic activity at very low doses in the form of Lipo-PEG-KillerTRAIL, where the reduction in tumor volume is significant even 15 days after the end of the treatment. Lipo-PEG-KillerTRAIL increases the therapeutic efficacy of the KillerTRAIL protein by 46%. The therapeutic protocol provides for the daily administration of 0.04 mg / kg (corresponding to the KillerTRAIL protein) for 7 days (therefore equivalent to 0.28 mg / kg of Lipo-PEG-KillerTRAIL equivalent to the amount of native protein) and the evaluation of the variation in tumor volume. from time 0 (first injection) for the next 15 days starting from the seventh day of treatment, for a total of 21 days. The experimental results obtained show that Lipo-PEG-KillerTRAIL increases compared to native KillerTRAIL:

1) The therapeutic efficacy of the native protein;
2) The therapeutic activity of the native protein after a single administration of Lipo-PEG-KillerTRAIL.

The therapeutic treatments on animal models, used forexperimentation *in vivo*, were stopped after 21 days of treatment as the animals, which develop the tumor, and

used as a control, and those treated with the KillerTRAIL protein, did not survive beyond 21 days of treatment, contrary to those treated with Lipo-PEG-KillerTRAIL (Example 8, Figure 10).

Furthermore, the conjugation with the PEG, which covers the external surface of the lipid *bilayer* of Lipo-PEG-KillerTRAIL, on the only cysteine residue present in the native protein in position 230 (Cys 230), prevents the trimerization of the pro-apoptotic TRAIL protein, and in particular KillerTRAIL. This procedure therefore allows not only to PEGylate the single monomer of the KillerTRAIL protein, but also to prevent its trimerization. This effect is evident in the activity and efficacy of Lipo-PEG-TRAIL in an absolutely unexpected way, guaranteeing the further advantage of a co-administration of TRAIL with lipophilic or amphiphilic anticancer drugs co-conveyed using the micellar aggregates obtained with the conjugated. The difference between Lipo-PEG-KillerTRAIL and KillerTRAIL was studied and, in case it is necessary to co-administer hydrophilic, lipophilic or amphipathic drugs, the data obtained were statistically significant (Figure 10, Example 8).

As an example, within the invention, the graph relating to the comparison of Lipo-PEG-KillerTRAIL and KillerTRAIL for the reduction of the volume of the tumor mass in mice implanted (*xenografat*) with colon carcinoma cells HTC116 (Figure 10, Example 8). These data demonstrate that Lipo-PEG-KillerTRAIL has a statistically significant effect on tumor reduction compared to KillerTRAIL.

The increase in therapeutic activity on *in vivo* mouse models, obtained on Balb / c mice implanted (*xenograft*) with colon carcinoma cells HTC116, for Lipo-PEG-KillerTRAIL compared to free KillerTRAIL (Example 8, Figure 10), and the enhancement of its therapeutic activity, possibly also in co-administration synergistically with hydrophilic, lipophilic and amphipathic drugs, or other amphipathic therapeutic peptides, such as those previously reported in the description of the invention, is further highlighted

by comparing the data obtained on the same models of Balb / c mice implanted (*xenograft*) with cells colon carcinoma HTC116 for the phospholipid-PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 13) and the phospholipid-PEG-KillerTRAIL, the PEG-KillerTRAIL and the free KillerTRAIL (Example 8, Figure 14).

Furthermore, the presence of KillerTRAIL, in monometric form, conjugated through the resiguo Cys-230 to the PEG chains through a maleimmiidic bond, favors the specific accumulation of the therapeutic proietin within the tumor tissue, and neither reduces its distribution in the organs. of the reticuloendothelial system and in the other tissues of the organism in which the KillerTRAIL protein does not have a selective action (Example 8, Figure 15 AC).

This reduction obtained on the volume of the tumor mass could be further improved by co-conveying within the drug formulation hydrophilic, lipophilic or amphipathic drugs, which could have a synergistic action with the native protein, reducing its resistance.

The increase in the *in vivo* therapeutic efficacy of Lipo-PEG-KillerTRAIL is an unexpected result, as the steric hindrance of the PEG could have prevented the binding of the native KillerTRAIL protein with the programmed cell death receptors, expressed on the surface of the membranes of the cells, and thus reduce their therapeutic effect.

The following examples are to be considered illustrative and not limitative of the scope of the present invention.

Examples

Materials

The KillerTRAIL was purchased from Enzo Lifescience (Florence, Italy)((http://www.enzolifesciences.com/ALX-201-073/killertrail-protein-soluble-human-recombinant/).

The phospholipid, 1, 2-dipalmitoyl-sn-glycero-3- phosphocholine monohydrate (DPPC) was purchased from Genzyme (Suffolk, UK) and / or Avanti Polar (USA); DSPE-PEG-maleimide 1,2-distearoyl-sn-glycerol-3 -phosphoethanolamine-N- (maleimide-(polyethylene glycol 2000)) (MW 2-200 kDa) was purchased from Avanti polar lipids (USA). Ethylene-diamino-tetraacetic acid (EDTA), phosphate buffer solution (PBS), the bicinchoninic acid assay *kit,* the ELISA TRAIL *kit,* picrylsulfonico acid, 5,5'-dithiobis (2-nitrobenzoate), Bradford reagent, cholesterol were purchased from Sigma-Aldrich Italy (Milan, Italy). the membranes used in dialysis experimental phase were purchased from Spectrum Laboratories Inc. (Eindhoven, The Netherlands). All aqueous solutions were prepared using deionized water (m illiQgrade, 18.2 MSZ) obtained using the Millipore MilliQ system (MA, USA). All the solvents and the remaining reagents used were selected from those specific for HPLC or with a degree of purity from those available in the catalog from Sigma-Aldrich (Milan, Italy) or Carlo Erba (Milan, Italy).

The products were characterized by high performance chromatography and by means of the inverse phase technique (C18 RP-HPLC) in which the protein was found to be stable with the aim of monitoring the progress of the reaction, determining its completion, quantifying the yield and purifying the derivatives obtained, as reported in the following section.

The products were analyzed by means of various types of colorimetric assays (BCA, Habeb, Bradford), to determine the concentration of the protein in solution and by means of assays at known concentration, to determine the interference of the PEG on these assays.

studies were performed on liposomes or Lipo-PEG-KillerTRAIL *Dynamic light scattering* (DLS)in order to obtain data on the particle size (size), on the polydispersion index (PDI) homogeneity of the dialysate system, and the surface charge of the nanovectors or zeta potential.

In vivo experiments of Lipo-PEG-KillerTRAIL were performed on mouse models (*xenograft*) of human liver cancer (HTC116) to determine the improvement of pharmacokinetic parameters, biodistribution and therapeutic antitumor activity in terms of tumor reduction and increase the survival of the products with respect to the native protein (Example 8, Figures 9 and 10).

Example 1: identification of the KillerTRAIL protein sequence

Characterization by Mass Mapping

The mapping of the entire sequence of the KillerTrail protein was carried out through MALDI-mapping approaches which allows to obtain reliable, reproducible and sensitive peptide maps of the protein under examination. Mass Mapping is a strategy used for the detailed structural characterization of a protein which consists in the construction of the peptide map of the protein itself. The mass signals observed in the MALDI spectra are of the MH + type and can be correlated to the peptides present in the protein sequence on the basis of their molecular weight, thus determining a peptide map of the protein itself. For this purpose a program, MS-Digest, is used where, once the known sequence of the protein is entered, a list of all the possible peptides that can be generated by hydrolysis of the protein with an appropriate enzyme with relative molecular weights is provided. The set of peptides found contributes to define the sequence "mapping" or the coverage of the amino acid sequence.

The presence of any unexpected abnormal signals on the basis of the amino acid sequence of the protein, were subsequently analyzed using direct tandem mass spectrometry techniques (MALDI-MS / MS analysis) and by LC-MS / MS and analysis of the extracted ion. The tandem mass spectrometry (MS / MS) allows to obtain a unique assignment of these signals, through the fragmentation of the unassigned peptides and the reconstruction of their amino acid sequence

To confirm the data obtained by MALDI-mapping, the peptide mixture was analyzed by LC-MS / MS and unassigned signals were evaluated using the extracted ion strategy. The possible m / z values of the peptide of interest were calculated assuming the following charge states: +1, +2 and +3. These calculated m / z values are used for searching within MS and MSMS tracks. Whenever the correspondence between a theoretical and an experimental data is found, the instrument software generates traces of ionic current with a peak of maximum intensity in correspondence with the retention time (RT) of the peptide ion. The perfect correspondence between the RTs of peptide ions relating to the same species that differ only in the state of charge is a confirmation of the presence of this peptide species. The set of peptides found contribute to define the Sequence Coverage.

Methods

For the structural characterization of the KillerTrail protein, various procedures and methods of analysis explained below have been carried out.

Hydrolysis with Trypsin

The sample was dried in Savant and resuspended in 50 mM Ammonium bicarbonate and then digested with trypsin at 37°C for 16 hours. The peptide mixture obtained, purified by C18 zip-tip, was dried in Savant and then resuspended in 0.2% TFA. A MALDI-MS analysis of the tryptic digest was performed by MALDI TOF-TOF 4800 Plus mass spectrometer.

Hydrolysis with GLU-C

The peptide mixture obtained from the previous trypsin hydrolysis was sub-digested with GLU-C in Ammonium bicarbonate 50 mM for at 37°C *over night* (E: S 1:50 w / w).

Molecular weight determination by ESI-MS analysis

300 µl of protein (833 pmol) was subjected to desalting by reverse phase high performance liquid chromatography (RP-HPLC). In detail, the instrument is an Agilent Technologies 1100 HPLC (Agilent Technologies, USA) with Phenomenex Jupiter C4 analytical column (250 mm × 2.0 mm, pore size 300 Å) and a linear gradient from 5% to 95% of solvent B (0.07 % TFA in 95% acetonitrile) in about 30min at a flow rate of 0.2ml / min. The sample was diluted in 0.2% TFA and injected through a calibrated syringe. From the observation of the chromatogram in Figure 1 it was possible to take the peak corresponding to the elution of the protein. The sample was dried in a SpeedVac system.

The sample was then resuspended in 50 µL of 50% ACN and 0.2% HCOOH and analyzed by ESI-MS mass spectrometry using the Q-TOF Premier system (Waters, Milford, MA, USA) with ESI source by direct syringe injection. calibrated at a flow rate of 10 µL / min. The mass spectrum was acquired in a range from 800 to 2500 m / z.

Raw data was processed using MassLynx 4.1 software (Waters, Milford, MA, USA). Figure 2 (AD) reports the ESMS spectrum obtained from which an experimental MW of 24.013.91 ± 0.94 Da was calculated.

MALDI-TOF analysis

The evaporated sample was solubilized in 10 µL of 0.2% TFA, of which 0.5 µL are were loaded on the MALDI plate together with an equal quantity of organic matrix (10 mg / ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 30% TFA 0.2%). The metal plate was inserted into the instrument, and the sample was impacted by the laser beam of the source with a predetermined intensity. The mass spectra obtained relate the intensity with the m / z ratio in a mass range from 500 Da to 5,600 Da.

The identification of the peptides was carried out on the basis of the mass values recorded in the spectra and the data they are summarized in Table 2.

Table 2. The table shows the mass values identified in the MALDI spectra and assigned to the corresponding peptides in the KillerTRAIL protein.

Position of the sequence	[MH]+ (Da)	Notes
158-161	533.16	
111-115	668.25	Nonspecific tryptic cut Tyr115
125-130	714.35	
144-149	715.41	
134-137	724.30	
80-85	**738.43**	
22-27	**742.35**	
56-61	776.38	
98-109 158-163	793.39	
92-97	801.36	Nonspecific tryptic cut Tyr91
70-76	852.48	Nonspecific tryptic cut Phe69
150-157	891.47	Nonspecific tryptic cut Tyr149
150-157	874.45	PYR GLU[1]
5-12	905.47	
28-36	925.50	
111-117	944.41	
57-64	978.44	
170-177	1009.42	
13-21	1037.56	PYR GLU[1]
13-21	1054.59	
111-118	1072.52	
90-97	1077.51	Nonspecific tryptic cut Tyr89
56-64	1106.53	

147-156	1126.55	Nonspecific tryptic cut Tyr146
28-38	1138.56	
89-97	1240.57	Nonspecific tryptic cut Tyr88
147-157	1254.64	Nonspecific tryptic cut Tyr146
98-107	1265.61	
39-50	1277.61	
159-169	1293.62	
65-76	1367.76	
119-130	1440.72	
143-155	1509.68	
86-97	1607.72	
85-97	1735.81	
147-161	1768.81	Nonspecific tryptic cut Tyr146
39-55	1802.87	
162-177	1898.9	
20-36	1904.81	
140-157	2065.96	
71-88	2125.90	
88-103	2178.04	
131-149	2212.02	Nonspecific tryptic cut Tyr149
70-88	2239.06	
138-157	2280.99	Nonspecific tryptic cut Tyr137
162-181	2351.05	Nonspecific tryptic cut Phe181
20-42	2533.23	
140-161	2580.16	

162-184	2626.18	Nonspecific tryptic cut Phe184
99-119	2692.20	
111-133	2807.25	
108-130	2851.39	
86-107	2854.4	
162-187	2895.34	
65-89	2917.37	Nonspecific tryptic cut Tyr89
108-133	3165.45	
57-85	3346.54	
77-103	3401.52	
77-107	3873.70	
65-107	5222.6	

[1]PYR GLU: Cycle of the N-terminal Gln peptide.

As can be seen, signals regarding peptides of the N-terminal segment of the protein are completely absent as they are linked to the Killer peptide and to the HIS Tag.

Some peaks in the spectrum of the KillerTrail protein are not justified by the Digest and these could be related to a peptide with the *linker* and the HIS Tag. To obtain a unique assignment of these signals it is necessary to resort to tandem mass spectrometry (MS / MS) experiments which allow to perform a fragmentation analysis of the unassigned peptides (MS / MS).

Tandem mass spectrometry allows to fragment a single peptide and to reconstruct its amino acid sequence. The m / z 2,549.19 peptide was then isolated in the collision cell and fragmented. The fragmentation spectrum obtained is shown in Figure 3.

LC-MS / MS analysis

The mixture of peptides coming from the hydrolysis with trypsin and Glu-C of the KillerTrail protein was also analyzed by LC-MS / MS with an LTQ Orbitrap mass spectrometer XL equipped with nano-HPLC (Thermo Fisher, USA). The peptide mixture was first concentrated and desalted with pre-column (C18 Easy Column L = 2 cm, ID = 100 mm, Thermo Fisher Scientific) and fractionated on a reversed phase C18 capillary column (L = 20 cm, ID = 7.5 µm, 3 µm, NanoSeparazion) working at a flow of 250 nl / min. Buffers A and B respectively had the following composition:

A (0.2% HCOOH, 2% ACN LC-MS Grade)

B (0.2% HCOOH, 95% ACN LC-MS Grade)

The peptide mixture was separated with a chromatographic gradient of 87 minutes. The analysis of the peptides was performed in "data dependent acquisition" (DDA) mode with a scanning interval from 300 to 1,800 m / z, followed by the MS / MS scan of the 5 most intense ions (top 5) obtained from the CID fragmentation (Collision Induced Fragmentation) and analyzed with ion trap.

Subsequently, the *raw* file obtained from the LC-MS / MS analysis was processed according to the Extracted Ion procedure to highlight the presence of the putative signal probably corresponding to the *linker* peptide (m / z = 2,549.19). The possible m / z value of the peptide of interest was calculated assuming the following charge states: +2 and +3. These m / z values calculated for the peptide of interest were used for searching within the MS and MS / MS traces. Figure 4A shows the TIC of the extracted ion corresponding to the peptide with m / z 2,549.19, and its fragmentation spectrum (Figure 4B).

The KillerTRAIL protein was subjected to MALDI analysis, after hydrolysis with trypsin and sub-digestion with GLU-C. The MALDI spectrum obtained was manually interpreted by associating a specific peptide to each peak. The process of assigning

each signal to a specific peptide allows to obtain the actual sequence coverage of the protein.

Figure 5 shows the sequence coverage obtained following hydrolysis of the KillerTrail protein.

The KillerTrail protein was characterized by MALDI analysis with a sequence coverage of 97.8%. In the spectrum of the protein there are no signals concerning the N-terminus of the protein.

The sample showed an experimental MW of 24,013.91 ± 0.94 Da which corresponds to the KillerTrail protein (theoretical MW: 21,607.1) with a mass difference of 2,406.81 Da. This calculated difference between the two species is represented by the *linker* and the His tag.

To obtain the sequence of the Killer peptide it was necessary to resort to tandem mass spectrometry (MS / MS) experiments using the MALDI TOF-TOF 4800 Plus mass spectrometer. The peptide Killercon m / z 2,549.19 considered the peptide corresponding to the Killer peptide is was subjected to fragmentation by MALDI-TOF / TOF analysis. To confirm this hypothesis the *raw file* obtained from the LC-MS / MS analysis was processed according to the Extract Ion procedure to highlight the presence of the Killer peptide and determine its amino acid sequence.

From the data obtained from the tandem mass spectrometry experiments in both MALDI-TOF / TOF and LC-MS / MS it was possible to reconstruct the amino acid sequence of the Killer peptide, with a sequence resolution, accuracy and precision of 90%, which is shown in Figure 6.

The Killer peptide consists of a 6 histidine tag (highlighted in yellow in the sequence), thepeptide *linker* (in green) and the first four amino acids of the Trail protein (in black) (Figure 6).

Through mass spectrometry approaches, the molecular weight of the KillerTRAIL protein was determined with a mass difference of 2,406.81 Da compared to the unmodified protein (commercial product). This calculated difference between the two species is represented by the *linker* and the His tag. Instead, by implementing a Mass Mapping strategy, it was possible to confirm the entire amino acid sequence with the exception of the N-terminal peptide linked to the Killer and the HIS-Tag. To obtain the sequence of the Killer peptide it was necessary to resort to tandem mass spectrometry (MS / MS) experiments using the MALDI TOF-TOF 4800 Plus mass spectrometer which allows to perform a fragmentation analysis of the peptide of interest (MS / MS).

The peptide with m / z 2,549.19, believed to be the putative peptide corresponding to the *linker* peptide, was subjected to fragmentation by MALDI-TOF / TOF. Furthermore, to confirm this hypothesis, the Raw File obtained from the LC-MS / MS analysis was processed according to the Extract Ion procedure.

In conclusion, it can be stated that the association of tandem mass spectrometry (MS / MS) experiments and a mass mapping method based on the extracted ion strategy allowed to obtain a reliable sequence of the peptide *linker*.

In light of these data it is possible to conclude that the mass analyzes have shown that the sequence of the Killer peptide corresponds to that reported in Figure 6 with a sequence resolution, accuracy and precision of 90%.

Example 2: Production of Lipo-PEG-KillerTRAIL

The lipid mixtures were solubilized in a conical bottom tube using 2 ml of chloroform / methanol (3: 1 v / v) and the organic solvent was subsequently removed from the lipid mixture using a Rotavapor® (mod. R110 of the Büchi company, Italy), under a continuous flow of nitrogen and / or a mechanical system for generating a vacuum until a lipid film is formed on the wall of the test tube. The excess organic solvent was eliminated from the formulations using a Büchi T51 vacuum dryer (Buchi, Italy)

connected to a vacuum pump and kept under operating conditions *over night.* The lipid film was subsequently hydrated with 1 ml of PBS solution at pH 7.4 containing the test protein KillerTRAIL (3.12 mg / ml). The suspension thus obtained was subsequently subjected to 3 cycles of stirring at 700 rpm for 3 minutes followed by as many heating cycles at 55°C in a thermostated water bath. The method used made it possible to obtain the formation of liposomal systems coated with PEG-KillerTRAIL. The systems thus obtained were subsequently kept at 45°C for 3 h to allow the lipid structure to stabilize and subjected to various extrusion cycles through polycarbonate membranes, using an extruder (Lipex Biomembranes, Northern Lipids Inc., Vancouver, BC, Canada). The systems were extruded through 2 polycarbonate filter membranes (Whatman Inc, USA) with pores of 400 and 200 nm respectively. For each extrusion cycle, 10 different steps were performed for each liposomal formulation. The pressure used for the process was respectively 430 and 900 kPa for polycarbonate membranes with pores of 400 and 200 nm. Post-conjugation was carried out according to the reaction scheme shown in Figure 7, at room temperature for 7 h and monitored by RP-HPLC (Figure 8).

Example 3: Conjugation efficiency of KillerTRAIL on liposomal systems

The conjugation efficiency of the KillerTRAIL protein was determined following disruption of liposomal systems by ultracentrifugation, using a Beckman ultracentrifuge equipped with a fixed angle TL S55 rotor (Optima TL Ultracentrifuge, Beckman, Coulter Inc., Fullerton, CA, USA) at 38,000 rpm at 4°C for 30 min.

The amount of KillerTRAIL released from Lipo-PEG-KillerTRAIL and present in the supernatant was determined using the BCA assay (Sigma-Aldrich, Milan, Italy). BSA (bovine serum albumin) was used as a control. The analyzes were performed in RP-HPLC (JASCO Europe srl, Cremella (LC), Italy) equipped with a PU 1580 binary pump module, degasser and diode array detector (or PDA detector) MD 1510 (JASCO Europe srl, Cremella (LC), Italy), using a C18 column, in reverse phase, Jupiter 250 × 4.6 mm,

5 µm (Phenomenex, USA). The amount of KillerTRAIL encapsulated was calculated using the following equation:

LE = (A - B) / A × 100

where, A is the known amount of KillerTRAIL used for the liposomal preparation, and B is the amount of KillerTRAIL encapsulated in the liposomal systems. All analyzes were performed in triplicate.

The reaction yield obtained was 42% higher for all the nanovectors produced, largely falling within the therapeutic window of the protein belonging to the pro-apoptotic TRAIL family, more particularly KillerTRAIL.

Example 4: Colorimetric analysis using standard assays

Colorimetric determination of protein concentration:

Habeeb assay

The quantitative determination of the amino groups of the KillerTRAIL protein on the liposomal surface and in the supernatants was calculated by the Habeeb colorimetric assay with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [78]. The TNBS is reacted under basic conditions with the amino groups of the samples to be analyzed and the chromophore that is formed is determined by spectrophotometry at a wavelength of 420 nm with a UV-Vis PerkinElmer LS25 spectrophotometer (PerkinElmer, Milan, Italy) .

The samples to be analyzed were prepared by dissolving the compounds in 975 µl of buffer containing 0.1 M sodium borate, pH 9.3. 25 µl of TNBS (1% w / v in DMF) were subsequently added to each sample.

The absorption at the wavelength of 420 nm of the sample is directly proportional to the concentration of amino groups, the concentration of which has been calculated on the basis of a calibration line obtained with solutions at known concentrations of glycyl-glycine (Gly-Gly) or mono-methoxy-PEG-amino (m-PEG-NH2).

BCA assay

In this procedure, bovine serum albumin (BSA) is the reference protein as its exact concentration can be determined by UV-Vis spectrophotometry.

The protein concentration was experimentally confirmed by colorimetric analysis using the BCA *Protein Assay kit* (Sigma-Aldrich SpA, Milan, Italy) and a BSA solution, with a known title, as *the standard* reference.

The *kit* uses, in the first phase of the reaction, the biuret method, which is based on the use of copper salts to measure the protein content; therefore, by adding Cu^{++} ($CuSO_4$) to the solution to be quantified, in an alkaline medium for sodium potassium tartrate, copper chelates Cuare formed^{+} with amino acid residues, which give a purple color. Two bicinconinic acid molecules chelate the cuprous cation causing the color of the solution to change from blue-green to violet. The intensity of the color is proportional to the protein concentration in a range from 20 to 2,000 µg / ml. This assay is based on a time-dependent reaction. Since the biuret assay measures in the range 5 mg / ml to 160 mg / ml, the *kit* presents a second phase of the analysis, with the colorimetric reaction of copper, which in the oxidation state +1, which chela two molecules of bicinconinic acid (BCA) giving a color that was quantified at a wavelength of 562 nm, with an ELISA reader ELX808 from BioTek Instruments (Winooski, Vermont, United States).

For the quantitative analysis, the calibration line with BSA was constructed and for each sample the analyzes were performed in triplicate.

Example 5:

***High performance liquid chromatography* (HPLC)**

chromatographic analyzes The chromatographic analyzes were performed with the *High Performance Liquid Chromatography system* Jasco Europe Srl (Cremella (LC), Italy), consisting of a PU-1580 binary pump system, a series of diodes (DAD) MD 1510 (Jasco Europe Srl, Cremella (LC), Italy). A Jupiter C18 Phenomenex (USA) analytical

column (250 × 4.6 mm, 5µm) was used for the RP-HPLC analysis. The eluents and the buffer solutions were filtered with a Millipore system (Benford, MA, USA) equipped with 0.22 µm filters and were sonicated with the ultrasonic system Ultrasonic Cleaner mod. 5210 Branson (Dambury, USA) for at least 30 minutes before their use in chromatographic analysis.

The protein solutions of KillerTRAIL and the liposomal suspensions obtained were analyzed by RP-C18 HPLC and the elution profile was obtained by UV-Vis spectrophotometry at a wavelength of 280 nm using a PerkinElmer UV-Vis LS25 spectrophotometer (PerkinElmer, Milan, Italy).

The mobile phase used for the evaluation of both proteins was H_2O and CH_3CN both containing 0.05% (v / v) of trifluoro acetic acid (TFA); the chromatographic separation was carried out by means of a Hgradient$_2$O / CH_3CN: at t = 0 minutes, 90% H_20 and 10% CH_3CN; at t = 25 minutes, 10% H_2O and 90% of CH_3CN; at t = 30 minutes, 90% H_2O and 10% of CH_3CN. The conjugation reaction of the KillerTRAIL protein on the surface of the preformed liposomes was monitored until the time required for the completion of the reaction.

Example 6: UV-Vis spectroscopy

The first preliminary evaluation of the KillerTRAIL protein was carried out with spectrometry using a UV / Vis 1 and 25 Perkin-Elmer spectrophotometer (Northwolk, CT, USA) thus determining its exact concentration. Starting from these considerations, a calibration line was constructed ($y = 4\ 10^{-8}\times +0.0213$; $R^2 = 0.9973$), on which to base the subsequent evaluations of the native KillerTRAIL protein. Starting from the data in the literature (Expasy Data-protein), we know that the molar extinction coefficient (ε) of the protein fraction 95-281 is 27.390 (Abs 0.1% (1 g / l) = 1.274, assuming that all residues of cysteine are in a reduced form, λ = 280 nm).

Example 7: *Dynamic Light Scattering*

Photo correlation spectroscopy, or *Dynamic Light Scattering* (DLS), is one of the most used methods to determine the size of the liposomal vectors present in the suspension of analyzed particles.

The dynamic light *scattering* technique was used to evaluate the average size of Lipo-PEG-KillerTRAIL, the polydispersion index or PDI, which gives an indication of the dimensional homogeneity of the system, and the zeta potential. The instrument used for the DLS analyzes was the Zetasizer Nano ZS (Malvern, UK), correlated with a solid phase *laser* photodiode with a nominal power of 4.5 mV, capable of operating at a wavelength of 670 nm. The *scattering* measurements were performed with a *back scattering* angle 173º. To obtain the values of the average dimensions and polydispersion indices of the correlograms (autocorrelation function) of the various samples; the third order function was employed. The samples were loaded into quartz cuvettes and diluted with sterile double-distilled water, filtered with polystyrene filters with *cut-off* 0.22 µm (Wathman Inc., Clifton, Nj USA) to avoid *multiscattering* phenomena during the analysis.

The zeta potential measurements of the systems were carried out using a *Doppler laser* anemometer capable of measuring the average electrophoretic mobility of particles in Brownian motion. The Smolucowisky constant F (Ka) with an average value of 1.5 was used to measure the zeta potential of the Lipo-PEG-KillerTRAILs as a function of their electrophoretic mobility. The measurements (Table 3 and 4) were made using a He-Ne *laser* at a wavelength of 633 nm with a nominal power of 5 mW.

Table 3. Physico-chemical characterization of the unilamellar and multilamellar liposomal system.

Formulations	Formulations	Formulations	Formulations
Dimensions (nm)	Dimensions (nm)	Dimensions (nm)	Dimensions (nm)

PDI1	PDI1	PDI1	PDI1

Table 4. Physico-chemical characterization of Lipo-PEG-KillerTRAIL by post conjugation of unilamellar and multilamellar KillerTRAILs.

Formulations	**Dimensions (nm)**	**PDI[1]**	**Zeta potential (mV)**
Lipo-PEG-KillerTRAIL, extruded	230 ± 1.23	0.243 ± 0.01	33.8 ± 2.25
Lipo-PEG-KillerTRAIL, multilamellar	443 ± 2.52	0.886 ± 0.06Polydispersion	32.4 ± 1.14

[1]PDI =index.

Example 8: *In vivo* evaluation of KillerTRAIL and Lipo-PEG-KillerTRAIL

The animals used for the experiment are male Sprague Dawley (SD) rats with an average weight of 220-250 g of 4-5 weeks and BALB / c nude mice (20 -25 g, 4-5 weeks) purchased from Charles River Laboratories (Germany). The animals were kept in the enclosure under controlled conditions, divided into groups of 6-8, with cycles of 12 h light / dark, with food and water as needed (*ad libitum*) for 2 weeks. The procedures involving the use of animals and their care have been conducted in accordance with institutional guidelines that comply with national and international laws and policies (European Community Council Directive, of 24 November 1986, 86/609 / EEC) .

Pharmacokinetic studies of KillerTRAIL and Lipo-PEG-KillerTRAIL

The pharmacokinetic characteristics of the KillerTRAIL protein and Lipo-PEG-KillerTRAIL were studied in rats using the intraperitoneal (ip) route of administration. The animals were cannulated through the jugular vein one day before the experiments. They were randomly divided into 5 groups (n = 5): 3 groups for the KillerTRAIL and 2 random groups for the study of liposomal derivatives (Figure 9).

Subsequently, the dose of KTRAIL protein equivalent to 10 µg (0.04 mg / kg) or the molar equivalent of liposomal derivatives in PBS (pH 6.0) was administered. The plasma samples taken at different times, after administration, were centrifuged and stored at -80°C until the time of analysis. Concentrations of KillerTRAIL in rat plasma samples were measured using the *kit* commercial KillerTRAIL ELISA(BioSource International, Inc., USA). Pharmacokinetic parameters (Table 5) were calculated from plasma concentration profiles by non-compartmental model analysis.

Table 5. Comparison of pharmacokinetic parameters after administration of KillerTRAIL and Lipo-PEG-KillerTRAIL in rats (10 µg / rat [P1] [MVP2] iv; 0.04 mg / kg) (n = 5).

Pharmacokinetic parameters	KillerTRAIL	Lipo-PEG-KillerTRAIL
AUC (ng * min / mL)	17,590.99 ± 71.34	69,415.74 ± 1,524.30
$T_{1/2}$ (min)	10.50 ± 1.03	297.65 ± .11.32
Clearance (mL / min)	2.27 ± 0.04	0.57 ± 0.08

AUC: Area under the curve from time 0 to infinity; $T_{1/2}$: Plasma half-life.

Antitumor activity of KillerTRAIL and Lipo-PEG-KillerTRAIL

The antitumor effect of Lipo-PEG-KillerTRAIL was studied on mouse models xenographed with HCT116 colon carcinoma cells. Seven days after inoculation, the mice were treated for seven days with KillerTRAIL and the liposomal derivative (50 or 100 µg / mouse / day[P3], KillerTRAIL equivalent). Thereafter, tumor volume was monitored for 21 days every 5 days. Mice were treated with free LIPO-PEG-KillerTRAIL or KillerTRAIL (100 mg / mouse KillerTRAIL equivalent) via the tail vein (Figure 10). Tumor volume was monitored for 24 days. This parameter was calculated using the longitude (L) and transverse diameter (W) of the tumor using the following formula:

$V = (L \times W2) / 2$

Tumor growth inhibition (TGI) values were calculated using the following formula:

TGI = (1 - treated TV / control TV) × 100

Where TV is the volume of the tumor.

Evaluation *In vivo* of KillerTRAIL, PEG-KillerTRAIL and phospholipid-PEG-KillerTRAIL

The animals used for the experiment are male Sprague Dawley (SD) rats with an average weight of 220-250 g of 4-5 weeks and BALB / c nude mice (20-25 gr, 4-5 weeks)

purchased from Charles River Laboratories (Germany). The animals were kept in the enclosure under controlled conditions, divided into groups of 6-8, with cycles of 12 hours of light / dark, with food and water ad libitum for 2 weeks. The protocols for animal experimentation and their welfare were conducted in compliance with institutional guidelines that comply with national and international laws and policies (European Community Council Directive, of 24 November 1986, 86/609 / EEC).

Evaluation of the pharmacokinetic parameters of KillerTRAIL and phospholipid-PEG-KillerTRAIL

The pharmacokinetic parameters of KillerTRAIL and phospholipid-PEG-KillerTRAIL were studied in Winstar rats previously housed and treated with the different formulations administered intraperitoneally (ip). The rats were cannulated through the jugular vein one day before the experiments, randomly divided into 6 groups (n = 6): 3 groups for the KillerTRAIL and 3 groups for the phospholipid-PEG-KillerTRAIL.

Subsequently, a dose of KillerTRAIL equivalent to 10 µg (0.04 mg / kg) or the molar equivalent of phospholipid-PEG-KillerTRAIL was administered, where both formulations were solubilized in PBS (pH = 6.0). Plasma samples were collected at different incubation times (15, 30, 60, 240 minutes), centrifuged and stored at -80°C until analysis. The plasma concentrations of KillerTRAIL, obtained from both pharmaceutical formulations, were quantified using the *kit* commercial KillerTRAIL ELISA(BioSource International, Inc., Fisher Scientific, USA). Pharmacokinetic parameters were calculated from plasma concentration profiles by non-compartmental model analysis (Table 5).

Table 5. Pharmacokinetic parameters of KillerTRAIL and of the phospholipid-PEG-KillerTRAIL conjugate.

Pharmacokinetic parameters

Sample	t1 / 2 (h)	Cmax (µg / ml)	tmax (h)	Vd (ml)	AUC (µg / ml × h)
KillerTRAIL	1.3 ± 0.12	1.3 ± 0.17	-	13.0 ± 0.19	11.3 ± 0.05
Phospholipid-PEG-KillerTRAIL	39.24 ± 0.26	1.55 ± 0.11	-	56.5 ± 0.21	52.34 ± 0.02

***In vivo* antitumor activity of KillerTRAIL, PEG-KillerTRAIL and phospholipid-PEG-KillerTRAIL**

The *in vivo* antitumor effect of KillerTRAIL, PEG-KillerTRAIL and phospholipid-PEG-KillerTRAIL was studied in nude mice xenographed with HCT116 colon carcinoma cells (3×10^6 cells / mL). Seven days after inoculation, when an optimal tumor mass size was reached, BALB / c nude mice were treated for seven days with KillerTRAIL, PEG-KillerTRAIL and phospholipid-PEG-KillerTRAIL (50 or 100 µg / animal / day [P4], equivalent to one dose of KillerTRAIL). Over the next 21 days, every 5 days, the volume of the tumor mass was measured. KillerTRAIL, PEG-Killer and phospholipid-PEG-KillerTRAIL were administered intravenously (iv) through the tail vein of the animal. Tumor volume was measured overall for 24 days. This parameter was calculated using the longitude (L) and the transverse diameter (W) of the tumor according to the following formula:

$V = (L \times W2) / 2$

Tumor growth inhibition (TGI) values were calculated using the following formula:

TGI = (1-TV sample / TV control) × 100

Where TV is the volume of the tumor.

Accumulation intratumoral and biodistribution *in vivo* of KillerTRAIL and phospholipid-PEG-KillerTRAIL

The biodistribution was performed on nude mice *xenografted* with HCT116 colon carcinoma cells (3×10^6 cells / mL). The cells were inoculated subcutaneously into the femur of BALB / c nude mice. The tumor mass grew within a week. The mice were treated for four days with a single dose (injected ip) of KillerTRAIL and phospholipid-PEG-KillerTRAIL and 96 h after injection were sacrificed. The tumor masses and the various organs (liver, lung, kidney, heart, brain, muscle, bone, pancreas, bladder, stomach, small intestine, colon, spleen) were surgically removed, collected, treated and stored as previously reported (see paragraph on the study of pharmacokinetics). The supernatants were tested using the commercial ELISAfor KillerTRAIL *kit* (BioSource International, Inc., Fisher Scientific, USA). The animals were divided into 2 groups (n = 8) and the assessment of intra-tumor accumulation and *in vivo* biodistribution was performed using KillerTRAIL and phospholipid-PEG-KillerTRAIL, in particular DSPE-PEG-KillerTRAIL, at different times of incubation (15, 30, 60, 240 minutes). The analyzes were performed in duplicate for both the KillerTRAIL formulation and the phospholipid-PEG-KillerTRAIL formulation (Figure 15 AC). The experimental data obtained are the mean of 2 different analyzes ± the standard deviation.

Example 9: MALDI-TOF mass spectrometry (*Matrix-Assisted Laser Desorption Ionization - Timeof Flight*)

MALDI-TOF mass spectrometry, an analytical technique that allows to determine with exactness and precision the molecular weight of macromolecules of biological interest and to determine their structure as a function of the mass / charge ratio was used to characterize the chemical structure and molecular weight of DSPE-PEG-KillerTRAIL. DSPE-PEG-maleimide is a commercial product and the mass spectrum is in line with the technical specifications of the product as reported below. The mass spectra were

obtained with the REFLEXinstrument *Time of Flight* (AB Sciex 4800 plus MALDI-TOF *Analyzer*) equipped with a *SCOUT* ion source capable of operating in positive linear mode. The ions generated by a pulsed beam of UV radiation, produced by a *laser* (nitrogen laser, wavelength of 337 nm), were accelerated to 25 kV. A saturated solution of synapinic acid in H_2O / CH_3CN (1: 1 v / v) was used as a reference solution during the analysis and added to the samples previously solubilized in aqueous solution containing TFA (0.1% v / v) with a ratio of 1: 1 v / v. For analytical purposes only, the DSPE-PEG-maleimide 2 kDa and the DSPE-PEG-KillerTRAIL were then analyzed. The data obtained from the MALDI-TOF analysis demonstrated the formation of the conjugate between the PEG and the KillerTRAIL at the end of the reaction process. The analysis was performed on DSPE-PEG-maleimide 2 kDa (Figure 11A) whose molecular weight was found to be 2,964.2 Da and DSPE-PEG-KillerTRAIL (Figure 11B) whose absolute molecular weight was found to be 24,018.2 Da.

Example 10: Electrophoretic analysis SDS PAGE (Electrophoresis on polyacrylamide gel)

Electrophoresis is an analytical technique that allows to separate charged molecules, based on their different mobility in a specific electric field. The proteins have been denatured with sodium-dodecyl-sulfate (SDS), an anionic detergent that binds the proteins themselves with high affinity (one molecule of SDS for every two amino acids (aa) approximately) and gives them a net charge proportional to the mass of the protein. itself. In this way the effective charge of the polypeptide chains can be neglected during the analysis and the only effective charge is that obtained on the sample after ionization with the SDS.

Since all proteins have been previously denatured and have the same mass / charge ratio, their electrophoretic mobility depends exclusively on the molecular weight and porosity of the gel, which acts as a sieve (Figure 12).

For the electrophoresis of the protein samples a running acrylamide gel was used to 12%(w / v), and a gel of acrylamide pre-travel *(stacking* gel) to 4% (w / v). The instrument used is a Mini-PROTEAN® Electrophoresis System (Bio-Rad, Laboratories, Inc., USA) equipped with a vertical cell, connected to a current generator. The percentage of polyacrylamide of 10% (w / v) in the gel was used because it has the best technical characteristics to separate the samples under examination. For the electrophoretic run 10 µg of protein were loaded, in a volume of 15 µL. Each sample was denatured with 15 µL of "sample buffer" consisting of: TRIS 250 mM, pH 6.8; 2% (v / v) SDS, 25% (v / v) beta-mercapto-ethanol, as a reducing agent to break any sulphide bridges present in the native protein; bromophenol blue 0.1% (v / v); 10% (v / v) glycerol. Before being loaded on the acrylamide gel, the samples were denatured at 100°C for at least 5 minutes after loading in the "*sample buffer*". The electrophoretic run was conducted at 100 mV for at least 1h in a run buffer consisting of: TRIS 1 mM, pH 8.3; glycine 960 mM; SDS 0.1% (v / v); deionized water.

At the end of the stroke, to observe the separation of the proteins, the acrylamide gel was reacted with a dye solution: 0.1% (w / v) *Coomassie Brillant Blue*, 40% (v / v) ethanol, 10% (v / v) of acetic acid. The acid-ethanol mixture has the function of precipitating the proteins on the gel and fixing the dye. Finally, the acrylamide gel was bleached to remove the excess dye and mark only the one remaining bound to the KillerTRAIL. The conjugation reactions leading to the formation of phospholipid-PEG-KillerTRAIL, in particular, DSPE-PEG-KillerTRAIL, were monitored by SDS-PAGE (Figure 12).

The polyacrylamide gel was then analyzed using the Molecular Imageroptical system® (ChemiDocTM, Biorad Laboratories Inc., USA). The electrophoretic chamber Horizon 58 (Life Techonologies Horizontal Gel Electrophoresis System, USA) was used for the electrophoretic evaluation.

Example 11: Statistical

analysis Statistical analysis of experimental pharmacokinetic data, and antitumor effects were obtained using the *t-Student* index. Avalue p <0.05 was considered statistically significant. All obtained values represent the mean of different measurements ± the standard deviation (SD).

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CLAIMS

1. Nanovector consisting of a liposomal system comprising one or more conjugates consisting of a polyethylene glycol (PEG) chain linked at one end to a phospholipid and at the other end to a pro-apoptotic protein belonging to thefamily of *tumor necrosis factor* cytokines, the binding of polyethylene glycol with the protein being a covalent bond on a cysteine residue (-SH) of the sequence of said protein provided by a thiol reactive group present on the polyethylene glycol.

2. Nanovector according to claim 1 wherein the cysteine residue is in position 230 of the peptide chain of the protein sequence in its monomeric form.

3. Nanovector according to any one of claims 1-2 wherein the pro-apoptotic protein is choice between: KillerTRAIL, TRAILR1 (DR4; Apo2; CD261; 5 TNFRSF10A) [1], TRAIL-R2 (DR5, KILLER, TRICK2A, 10 TRICK2B, CD262, TNFRSF10B), TRAIL-R3 (DcR1, LIT, TRID, CD263, TNFRSF10C), TRAIL-R4 (DcR2; TRUNDD; CD264; TNFRSF10D), and osteoprotegerin (OPG; OCIF; TNFRSF11B), LZ-TRAIL, IZ-TRAIL, FLAG / His-TRAIL, TRAIL-R1 (Mapatumumab), TRAIL-R2 (Lexatumumab; HGS-TR2J; Apomab, AMG655, LBY135; CS-1008 or 25 human TRA-8), TNFSF10, TL2; APO2L; CD253; Apo-2L, LTα, KillerTRAILβ, LTβ, ligands of CD20; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), TNFSF10, TL2; APO2L; CD253; Apo-2. Nanovector according to claims 1-3 wherein the polyethylene glycol has an average molecular weight selected from the following ranges: between 200 Da and 30,0000 Da, between 3,000 and 100,000 Da, between 5,000 Da and 80,000 Da, between 10,000 Da and 40,000 Da , between 2 and 200 kDa, between 10 and 50 kDa.

4. Nanovector according to any one of claims 1-3 wherein the polyethylene glycol is an alkoxy-polyethylene glycol, preferably a methoxy-polyethylene glycol.

5. Nanovector according to any one of claims 1-4 wherein the protein is a monomeric protein and comprises the sequence Seq ID No 1 TSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSG HSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPILLMKSAR NSCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG.

6. Nanovector according to the previous claim in which the Seq ID No 1 is conjugated to the peptide sequence Seq ID No 2: HHHHHHYLMYGTVTLGY through the head residues Seq ID No 4: TSEE of said Seq ID No 1.

7. Nanovector according to any of claims 1-6 wherein the protein is monomeric KillerTRAIL having Seq ID No 3: HHHHHHYLMYGTVTLGYTSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSK NEKALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQ YIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFF GAFLVG

8. Nanovector according to claim 5 wherein the protein is a mutant comprising a polypeptide having an amino acid sequence with at least 50%, 52%, 54%, 56%, 58%, 60%, 62%, 64%, 66%, 68%, 70%, 72%, 74%, 76%, 78%, 80%, 82%, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98%, 99% or greater sequence similarity with respect to one of the SEQ ID No 1, Seq ID No 2, Seq ID No 3, Seq ID No 4.

9. Nanovector according to any one of claims 1-8, in which the reactive group towards the cysteine residue is selected from: PEG-maleimide (PEG-MAL), PEG-orthopyridine disulfide (PEG-OPSS), PEG iodine-acetamide (PEG- IA) and PEG-vinyl-sulfone (PEG-VS), preferably, PEG-MAL and PEG-OPSS.

10. Nanovector according to any one of claims 1-9 wherein the protein is selected from LTα, KillerTRAILβ, LTβ, the CD20 ligands; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), KillerTRAIL, TNF-apoptosis induced ligand (KillerTRAIL), TNFSF10, TL2; APO2L; CD253; Apo-2L (Entrez Gene ID: 8743; access number NM_003810.2; UniProtKB / Swiss-Prot: P50591; UniProtKB / TrEMBL: Q6IBA9).

11. Nanovector according to any one of claims 1-10 wherein the protein is TRAIL or a mutant thereof, preferably KillerTRAIL.

12. Nanovector according to any one of claims 1-11, wherein the phospholipid is selected from: polyethylene glycol 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-

[methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dimyristol-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 750]}; N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 2000]}; N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 5000]}; 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [dibenzocyclooctyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-azido (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [succinyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [carboxi (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [maleimide (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-

phosphoethanolamine-N- [PDP (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [amino (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [biotinyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [cyanur (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [folate (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [folate (polyethylene glycol) -5000] (ammonium salt); 1,2-dystearoyl-sn-glycerol-3-phosphoethanolamine-N- [poly (ethylene glycol) 2000-N'-carboxyfluorescein] (ammonium salt) and polyethylene glycol: 1,2-dimyristyl-sn-glycol- 3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -750] (ammonium salt); 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -1000] (ammonium salt); 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -2000] (ammonium salt); 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -3000] (ammonium salt); 1,2-dimyristol-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt);

1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N- [methoxy (polyethylene glycol) -5000] (ammonium salt); N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 750]}; N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 2000]}; N-palmitoyl-sphingosine-1- {succinyl [methoxy (polyethylene glycol) 5000]}; 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [dibenzocyclooctyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [azido (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [succinyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [carboxy (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [maleimide (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [PDP (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [amino (polythylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [biotinyl (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [cyianur (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [folate (polyethylene glycol) -2000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [folate (polyethylene glycol) -5000] (ammonium salt); 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N- [polyethylene glycol) 2000-N'-carboxyfluorescein] (ammonium salt).

13. Nanovector according to any one of claims 1-12 wherein the phospholipid is distearoyl-phosphatidyl-ethanolamine (DSPE).

14. Nanovector according to one of claims 1-13 wherein the polymeric conjugate is radiolabelled with ^{125}I,^{188}Re, or ^{188}W.

15. Nanovector according to any one of claims 1-14 further comprising one or more lipophilic, hydrophilic and amphiphilic substances encapsulated in the aqueous core and / or in the hydrophobic or lipid *bilayer* and the protein is disposed on the outer surface.

16. Nanovector according to claim 15 in which the drugs are selected from: lipophilic drugs: paclitaxel, docetaxel, campothecin, etoposide, doxorubicin anhydrous base, irinotecan anhydrous base, *vascular endothelial growth factor* (VEGFR) *inhibitors* such as Cabozantinib, Nintedanib, Wnt

/ β -catenin *modulators* such as XAV-939 (CAS NO. 284028-89-3), ICG-001 (CAS NO. 780757-88-2), Hedgehog *inhibitors* such as SANT75, HPI1 Antibody or HIC0-4F9, PI3K (Phosphoinositide 3-kinases) / (Proteinkinase B) Akt / (mammalian target of rapamycin) mTOR *modulators* such as Rapamycin, Buparlisib; amphiphilic drugs: natural peptides such as ovine lactofericin, magainin 2, hCAP109–135 (comprising the C-terminal domain of human CAP18) and BMAP-28 from bovine myeloid cathelicidin, neural (N) -cadherins, His-Ala-Val (HAV) sequence, Ala-Val (HAV), cyclic peptide N-Ac-CHAVC-NH2 or ADH-1 (Exherin), Cyclic peptides containing the sequence His-Trp-Gly-Phe (HWGF), peptideRS-83277, myelopep-tide- 2 (MP-2), tyroserleutide (YSL), Lactoferricin, Angiotensin (1–7), Growth inhibitorypeptide (GIP), p53-derivedapoptotic peptide, Cilengitide, ATN-161, ADH-1 (Exherin), WX-360 (uPA), WX-360-Nle (uPA), ABT-510 (Throspondin-1), MDP, Tyroserleutide, Bestatin, AFPep, Hunter-killer peptide, P15 with potent antitumor activity; hydrophilic drugs: gemcitabine hydrochloride, doxorubicin hydrochloride, cisplatin, irinotecan hydrochloride, carboplatin, oxaliplatin, 5-fluorine uracil, microRNA, capecitabine hydrochloride, erlotinib; and / or combinations thereof.

17. Nanovector according to any one of claims 1-16 wherein the liposomal system comprises at least one of the liposomal formulations containing: doxorubicin hydrochloride, amphotericin B, Daunorubicin citrate salt, doxorucine hydrochloride, vincristine sulphate, mifamurtide, doxorubicin hydrochloride, verteporanf virosomal inactivated hepatitis A virus of RG-SB strain, surface antigen virosomal inactivated influenza virus A / California / 7/2009 (H1N1) pdm09 said derivative strain used NYMC X-181, cisplatin.

18. Nanovector according to any one of claims 1-17 for use in the therapeutic, diagnostic or theranostic field.

19. Nanovector according to any one of claims 1-18 for use in the treatment of solid and liquid tumors, inflammatory and autoimmune diseases characterized by the presence on the cell and / or tissue surfaces of apoptotic receptors of the TRAIL family, preferably KillerTRAIL, such as TRAILR1 (DR4; Apo2; CD261; 5 TNFRSF10A)], TRAIL-R2 (DR5, KILLER, TRICK2A, 10 TRICK2B, CD262, TNFRSF10B), TRAIL-R3 (DcR1, LIT, TRID, CD263, TNFRSF10C), TRAIL-R4 (DcR2; TRUNDD; CD264; TNFRSF10D), and osteoprotegerin (OPG; OCIF; TNFRSF11B), LZ-TRAIL, IZ-TRAIL, FLAG / His-TRAIL, TRAIL-R1 (Mapatumumab), TRAIL-R2 (Lexatumumab; HGS-

TR2J; Apomab, AMG655, LBY135; CS-1008 or 25 human TRA-8), TNFSF10, TL2; APO2L; CD253; Apo-2L, LTα, KillerTRAILβ, LTβ, ligands of CD20; CD40, CD30, CD27, OX40, 4-1BB, FasL and CD253 (TRAIL), TNFSF10, TL2; APO2L; CD253; Apo-2.

20. Nanovector according to any one of claims 1-19 for use in the treatment of Leukemia, Breast cancer, Prostate cancer, Lung cancer, Renal cancer, Pancreatic cancer, Alzheimer's, Melanoma, Myelodysplastic syndrome, Lymphomas, Liver cancer, Rhabdomyosarcoma, Glioma , Non-small cell lung cancer, Hepatitis B virus, Hepatitis C virus, Fatty liver disease not related to alcohol abuse, Liver fibrosis, Cirrhosis, Mesothelioma, Pulmonary arterial hypertension, Systemic sclerosis, Cervical cancer, Fibrosis lung, Asthma, influenza Virus, atherosclerosis, lupus erythematosus, multiple sclerosis, HIV, amyotrophic lateral sclerosis, Cytomegalovirus, rheumatoid arthritis, *Myasthenia,* gravis, pancreatic carcinoma, adenocarcinoma ductal of the pancreas and neuroendocrine tumors of the pancreas originating from cells of the of *islets of Langerhans,* Carcionoma non-small cell lung cancer, squamous cell lung cancer, Large cell lung cancer, Lung adenocarcinoma, Lung sarcomatosis, Lung lymphomas, Carcinoma of the uterine cervix, Squamous cell carcinoma of the uterine cervix, Adenocarcinoma of the uterine cervix, Mesothelioma, Mesothelioma of the lung (pleura), Mesothelioma of the heart (pericardium), Mesothelioma of the heart (pericardium) of the intestine (peritoneum), Mesothelioma of the testicles (tunica vaginale), rhabdomyosarcoma (embryonic and alveolar), Chronic lymphocytic leukemia, Gliomas, glioma subtypes, Glioblastoma, Anaplastic Astrocytoma, Diffuse Astrocytoma, Pylocytic Astrocytoma, Other Astrocytoma, and other.

21. Nanovector according to one of claims 1-20 for oral, parenteral, rectal, topical, vaginal, ophthalmic or inhalation use.

22. Composition comprising a nanocarrier according to one of claims 1-17 and at least one further element selected from therapeutically active, diagnostic agents, vectors, adjuvants, excipients and pharmaceutically acceptable stabilizers and relative mixtures.

23. Composition according to claim 22 wherein the therapeutically active agent is selected from lipophilic drugs: paclitaxel, docetaxel, campothecin, etoposide, doxorubicin anhydrous base, irinotecan base anhydrous, *vascular endothelial growth factor* (VEGFR) *inhibitors* such as Cabozantinib, Nintedanib, Wnt / β-catenin *modulators* such as XAV-939 (CAS NO. 284028-89-3),

ICG-001 (CAS NO. 780757-88-2), Hedgehog *inhibitors* such as SANT75, HPI1 *Antibody* or HIC0-4F9, PI3K (Phosphoinositide 3-kinases) / (Proteinkinase B) Akt / (mammalian target of rapamycin) mTOR *modulators* such as Rapamycin, Buparlisib; amphiphilic drugs: natural peptides with powerful antitumor activity; hydrophilic drugs: gemcitabine hydrochloride, doxorubicin hydrochloride, cisplatin, irinotecan hydrochloride, carboplatin, oxaliplatin, 5-fluorine uracil, microRNA, capecitabine hydrochloride, erlotinib; and / or combinations thereof.

24. Composition according to claim 22, wherein the therapeutically active agent is at least one selected from the liposomal formulations containing at least one of doxorubicin hydrochloride, amphotericin B, Daunorubicin citrate salt, doxorucine hydrochloride, vincristine sulfate, mifamurtide, doxorubicin hydrochloride, verteporanfine, iritaxin , virosomal inactivated hepatitis A virus of strain RG-SB, virosomal inactivated influenza virus surface antigen A / California / 7/2009 (H1N1) pdm09 said derivative strain used NYMC X-181, cisplatin.

25. Composition according to any one of claims 22-24 wherein the diagnostic agent is a radio-labeled diagnostic agent, preferably ^{125}I, ^{188}Re, ^{188}W and combinations thereof.

26. Composition according to any one of claims 22-25 further comprising at least one of buffers, antioxidants, preservatives, low molecular weight polypeptides such as, for example, compounds containing less than about 10 amino acid residues with a molecular weight in the range of 10 to 1,000 Da, hydrophilic polymers, amino acids, carbohydrates, sugars, salt-forming counter-ions, non-ionic surfactants, ion exchangers and related mixtures.

27. Composition according to claim 26 wherein the buffers are selected from tris-(hydroxymethyl) -amino-methane hydrochloride (Tris-HCl), 4- (2-hydroxyethyl) -1-piperazine-ethanesulfonic acid (HEPES), phosphate, citrate, other organic acids and their mixtures.

28. Composition according to one of claims 22-27 wherein the antioxidant is ascorbic acid, methionine or their mixtures.

29. Composition according to any one of claims 22-28 wherein the preservatives are selected from octadecyl-dimethyl-benzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl or benzyl alcohol, alkyl parabens (methyl or propyl paraben); catechol; resorcinol; cyclohexanol; 3-pentanol and related mixtures.

30. Composition according to any one of claims 22-29 further comprising at least one of polysaccharides, preferably methyl-cellulose or carboxy-methyl-cellulose, polyvinylpyrrolidone, polyacrylates, ethylene-propylene block polymers, polyethylene glycol and waxes.

31. Composition according to any one of claims 22-30 in the form of liposomes, patches, inhalation forms, nasal sprays and prolonged release preparations.

32. Composition according to any one of claims 22-31 for use in the therapeutic, diagnostic or theranostic field.

33. Composition according to any one of claims 22-32 for use in the treatment of solid and liquid tumors, inflammatory and autoimmune diseases characterized by the presence on the surfaces of the cells and / or tissues of apoptotic receptors of the TRAIL family, preferably KillerTRAIL.

34. Composition according to any one of claims 22-33 for use in the treatment of leukemia, breast cancer, prostate cancer, lung cancer, renal cancer, pancreatic cancer, Alzheimer's, melanoma, myelodysplastic syndrome, lymphomas, liver cancer, rhabdomyosarcoma, glioma , Non-small cell lung cancer, Hepatitis B virus, Hepatitis C virus, Fatty liver disease not related to alcohol abuse, Liver fibrosis, Cirrhosis, Mesothelioma, Pulmonary arterial hypertension, Systemic sclerosis, Cervical cancer, Fibrosis lung, Asthma, influenza Virus, atherosclerosis, lupus erythematosus, multiple sclerosis, HIV, amyotrophic lateral sclerosis, Cytomegalovirus, rheumatoid arthritis, *Myasthenia,* gravis, pancreatic carcinoma, adenocarcinoma ductal of the pancreas and neuroendocrine tumors of the pancreas originating from cells of the of *islets of Langerhans,* Carcionoma non-small cell lung cancer, squamous cell lung cancer, Large cell lung cancer, Lung adenocarcinoma, Lung sarcomatosis, Lung lymphomas, Carcinoma of the uterine cervix, Squamous cell carcinoma of the uterine cervix, Adenocarcinoma of the uterine cervix, Mesothelioma, Mesothelioma of the lung (pleura), Mesothelioma of the heart (pericardium), Mesothelioma of the heart (pericardium) of the intestine (peritoneum), Mesothelioma of the testicles (tunica vaginale), rhabdomyosarcoma (embryonic and alveolar), Chronic lymphocytic leukemia, Gliomas, glioma subtypes, Glioblastoma, Anaplastic Astrocytoma, Diffuse Astrocytoma, Pylocytic Astrocytoma, Other Astrocytoma, and other.

35. Composition according to any one of claims 22-34 for oral, parenteral, rectal, topical, vaginal, ophthalmic or inhalation use.

36. Process for preparing the nanovector according to any one of claims 1-17 comprising the stages following fundamentals:

(i) solubilize in an organic solvent an aliquot of one or more phospholipids selected from neutral, cationic and anionic and add an aliquot of a PEG functionalized phospholipid and chosen from: phospholipid-PEG adduct and phospholipid-PEG-protein conjugate, the phospholipid functionalized with PEG being in relationship with one or more phospholipids according to the quantity chosen in the *range* molar fraction0:10 - 10: 0;

(ii) dry the mixture prepared in step (i);

(iii) then rehydrate with water or with an aqueous solution of buffer at pH 5-8, placing the aqueous suspension under mechanical stirring;

(iv) extrude the liposomes obtained in step (iii).

37. Process according to claim 36 in which, if a phospholipid-PEG adduct is used to obtain the liposomes of stage (iv), said liposomes are then conjugated with the TRAIL protein or one of its mutants, in particular KillerTRAIL, by binding covalent between one end of the PEG and the thiol group (-SH) of the amino acid cysteine of the protein at position 230.

38. Process according to claim 36 in which, in the event that a phospholipid-PEG-protein is used to obtain the liposomes of stage (iv), said conjugate will have been previously prepared with a method comprising the following main steps:

- Prepare an aqueous solution buffered at a pH between 5 and 8 containing the protein in monomeric form;
- Add the phospholipid-PEG polymer adduct in a ratio of 1: 4 (protein: adduct) and keep stirring until the reaction is complete.

39. Process according to claim 36 in which, if neither the phospholipid-PEG adduct nor the phospholipid-PEG-protein conjugate is used to obtain the liposomes of stage (iv), steps (i) to (iv), the phospholipid-PEG-protein conjugate is then separately prepared as indicated in claim 36 and continues with the process steps indicated below:

- Disperse the phospholipid-PEG-protein conjugate in an isotonic solution at physiological pH to obtain nanoaggregates;
- then purify the nano-aggregates thus obtained;
- Mix the liposomes of the previous step (iv) with the nanoaggregates just prepared under mechanical stirring conditions and at a temperature between 18 and 75 ° C.

40. Process according to any one of claims 36-39 comprising a further freeze-drying step.

41. Process according to claim 40 in which the lyophilization step is preceded, followed or replaced by a step in which the nanocarrier is combined with one or more lipophilic, hydrophilic or amphiphilic drugs.

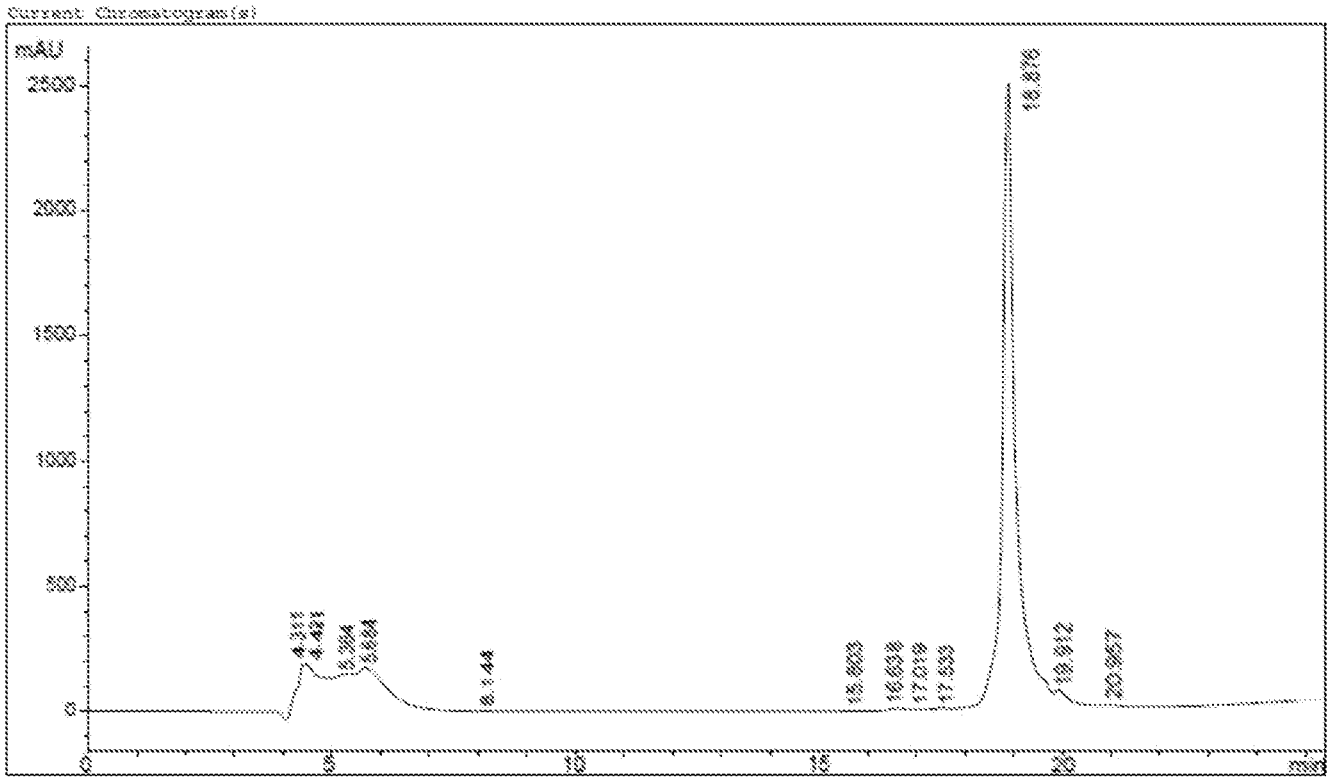

Figure 1

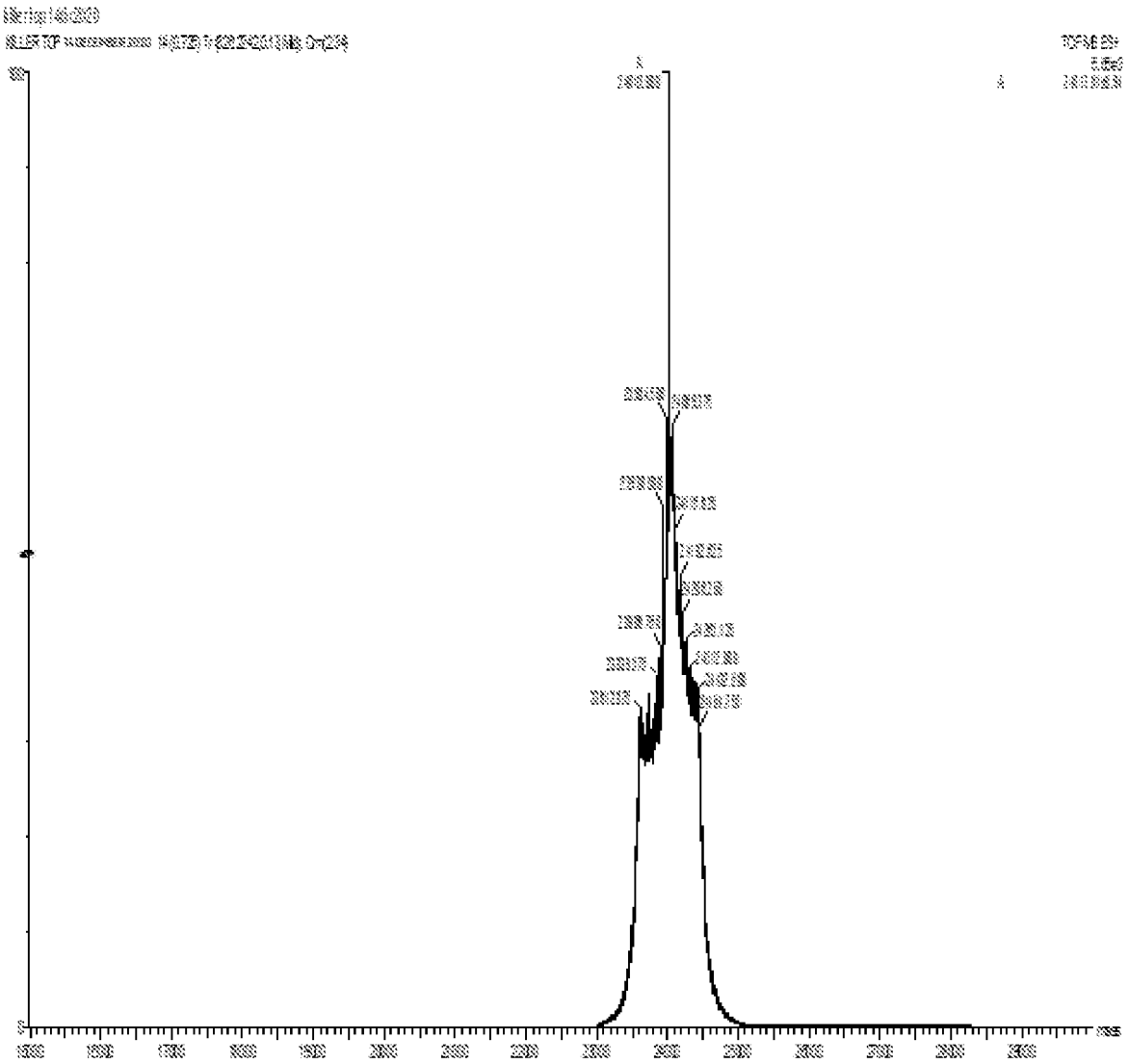

Figure 2A

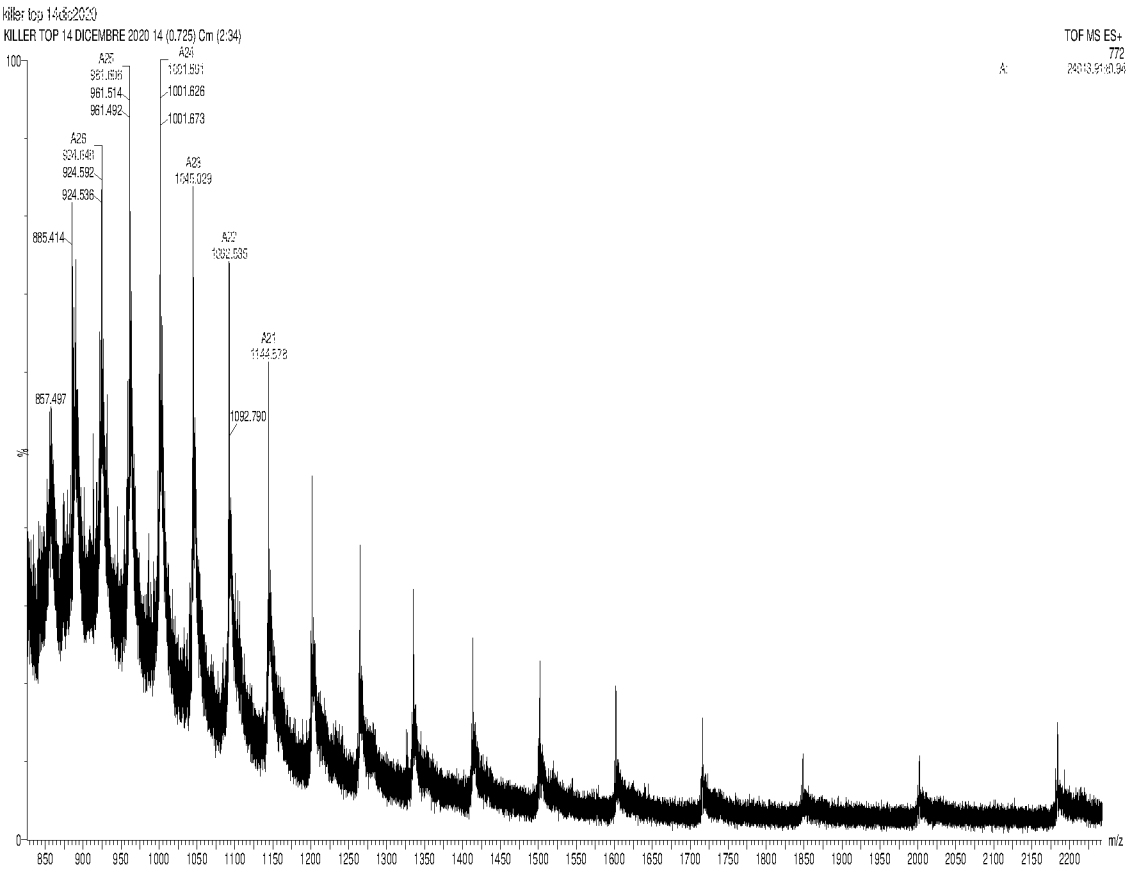

Figure 2B

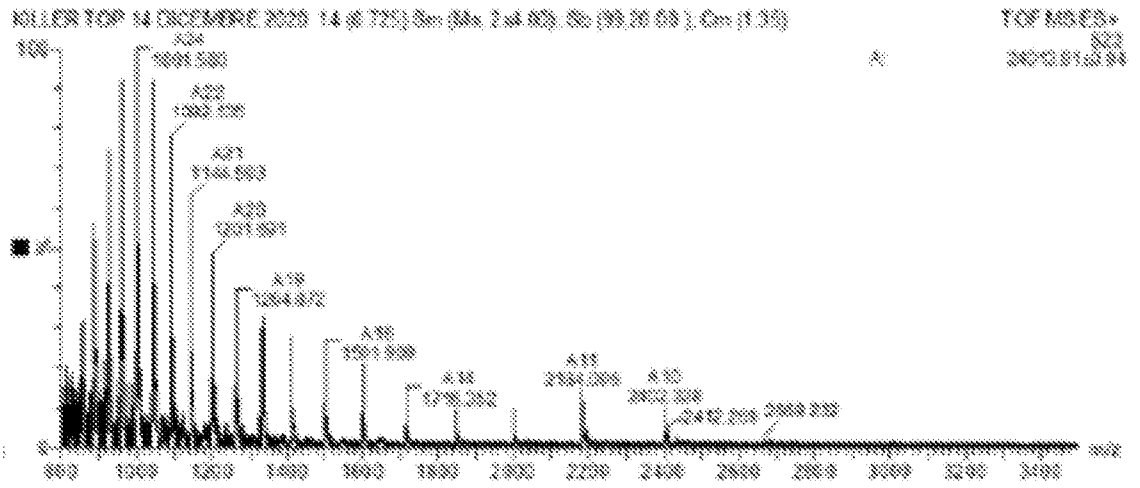

Figure 2C

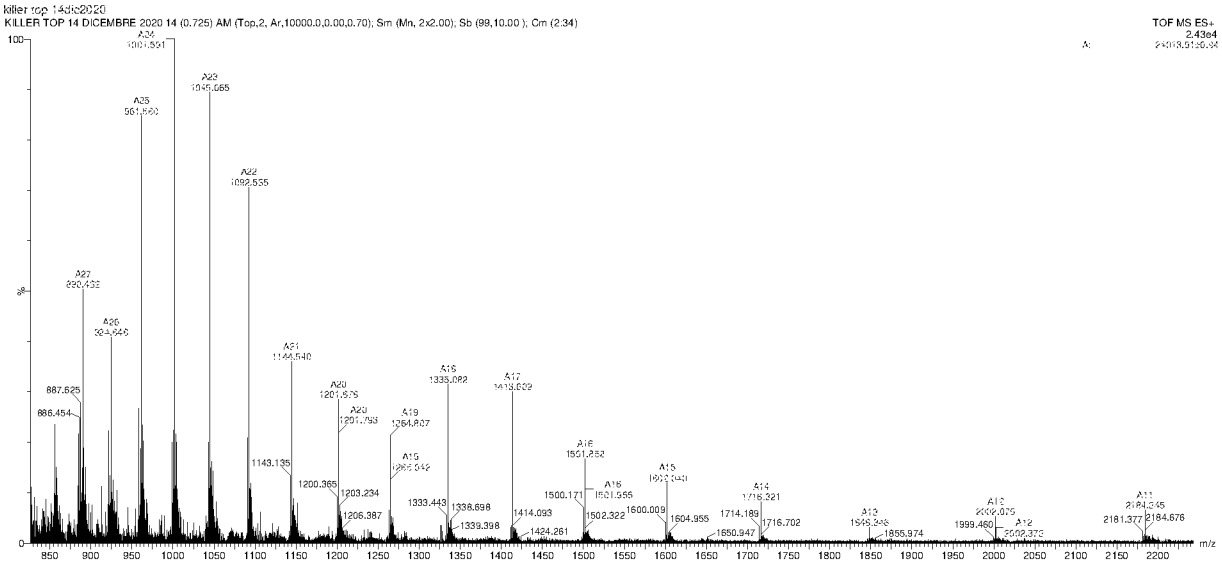

Figure 2D

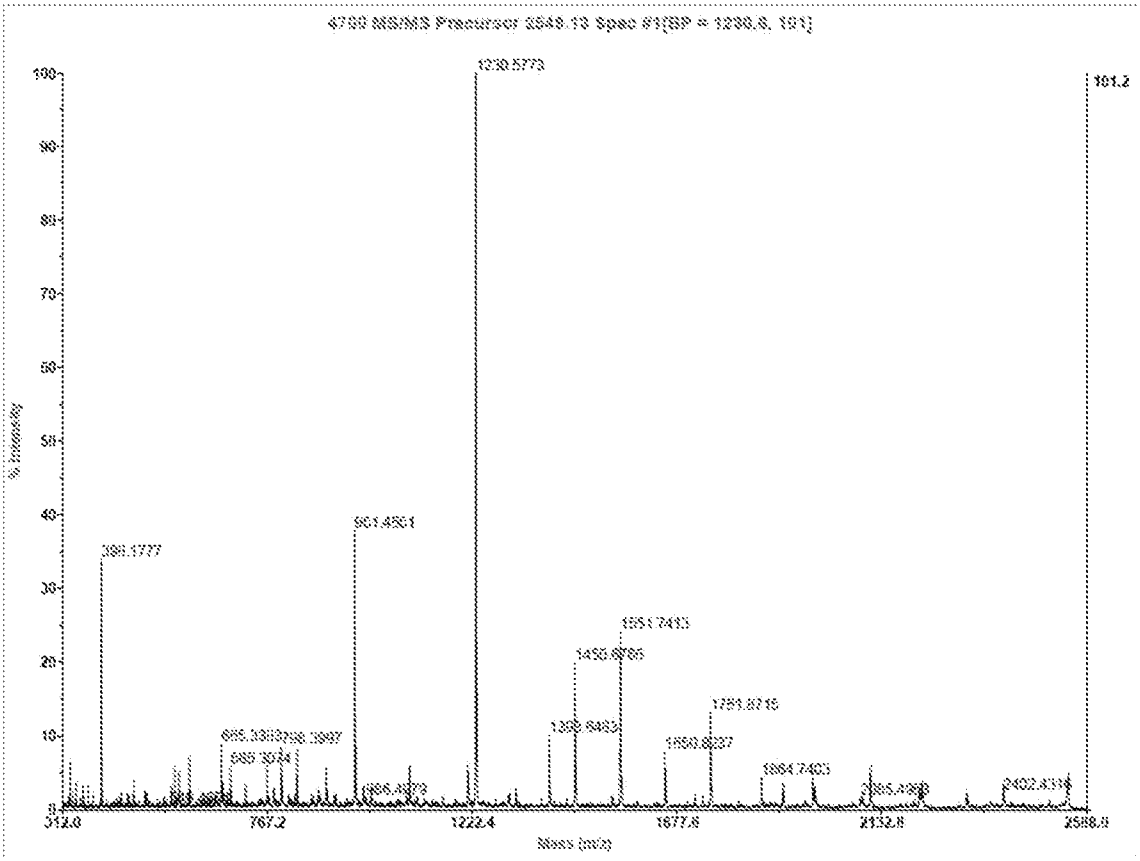

Figure 3

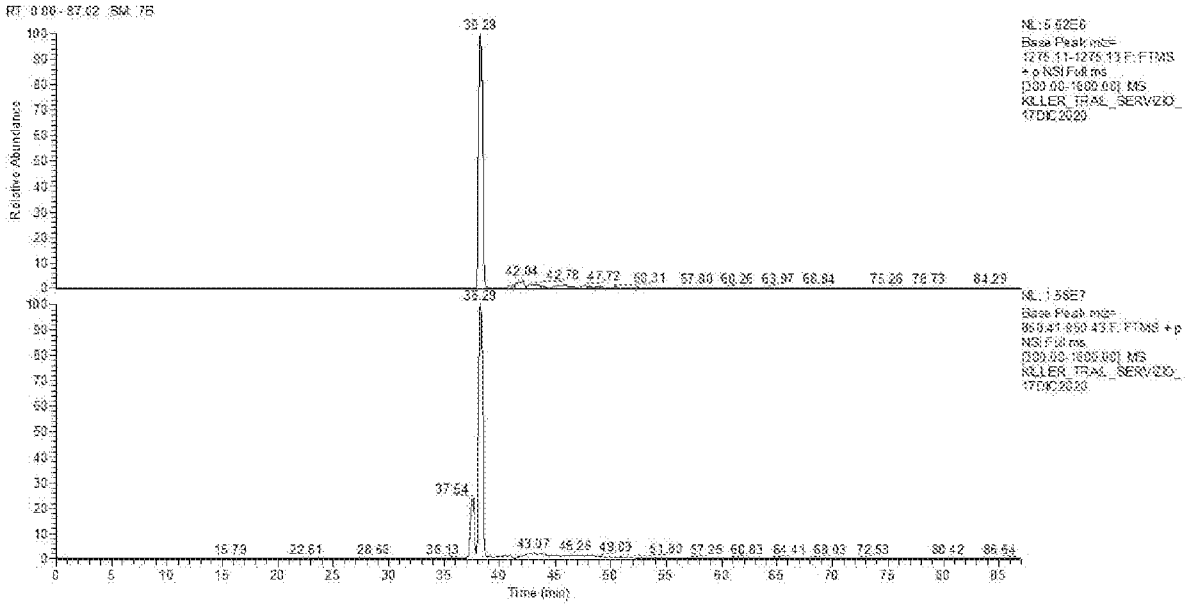

Figure 4A

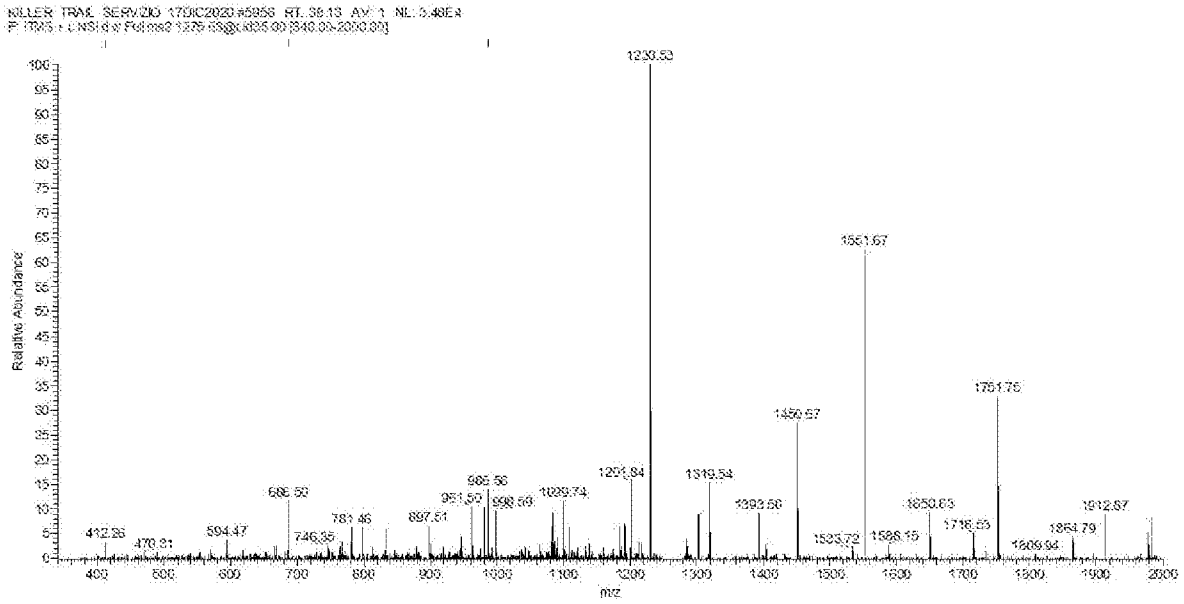

Figure 4B

Seq ID No 1: **TSEE**TISTVQ EKQQNISPLV RERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG

Figure 5

Seq ID No 2: HHHHHHYLMYGTVTLGY**TSEE**

Figure 6

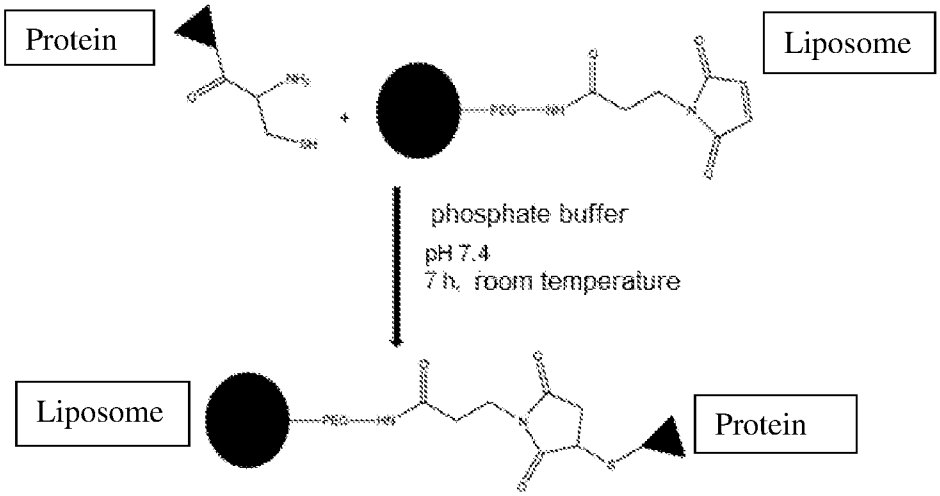

Figure 7

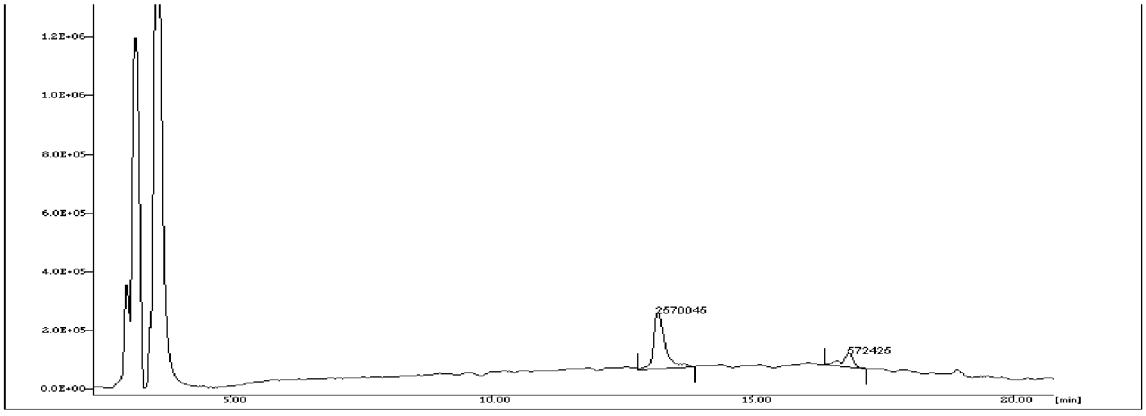

Figure 8

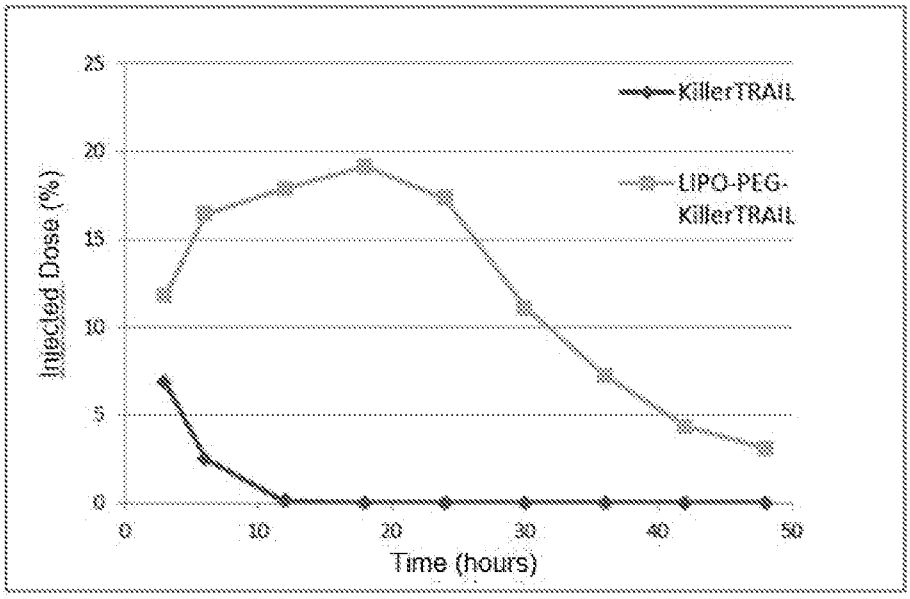

Figure 9

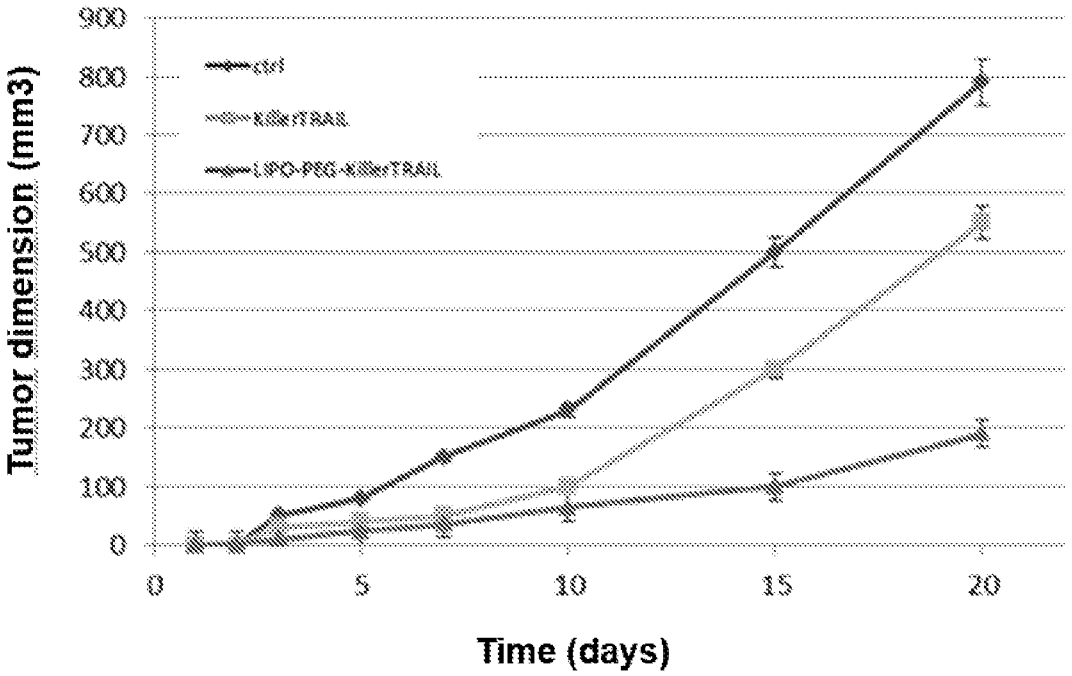

Figure 10

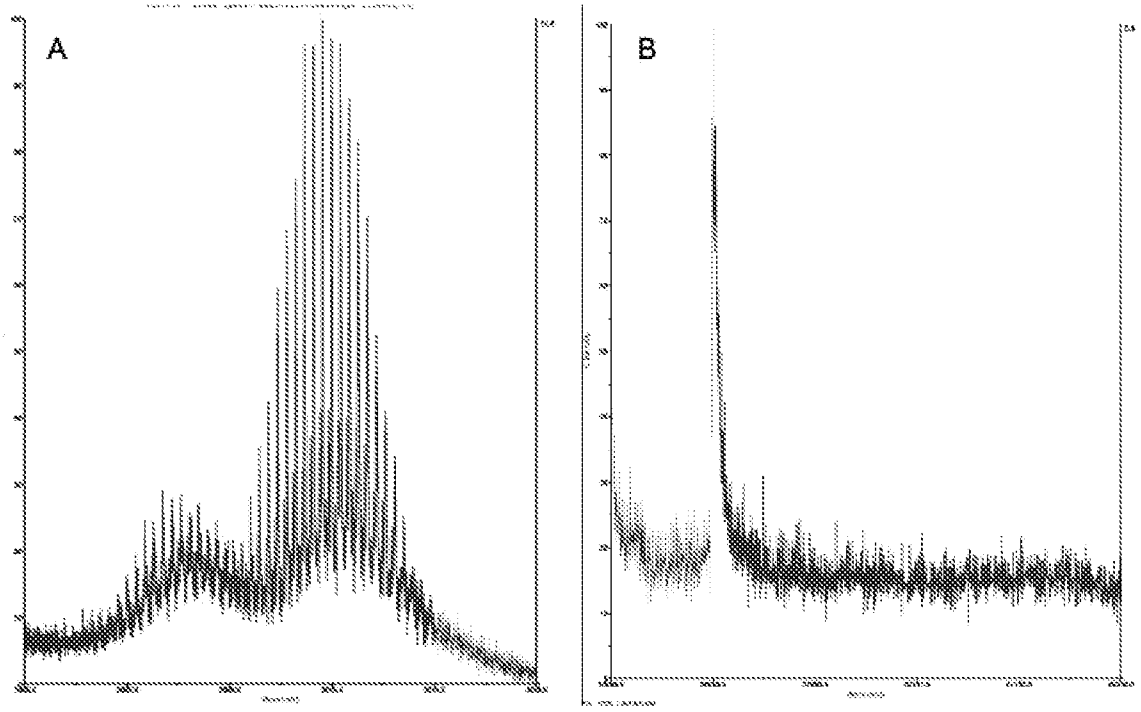

Figure 11

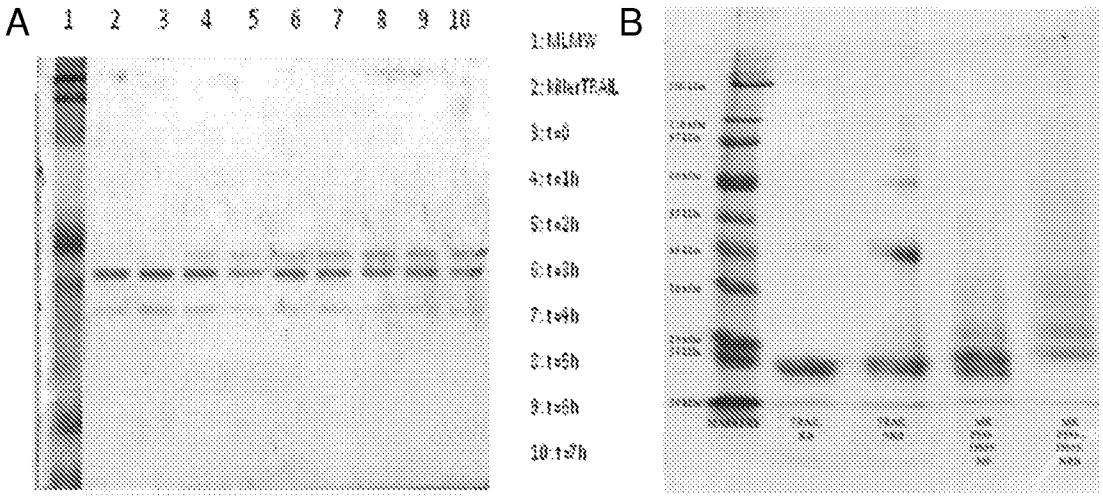

Figure 12

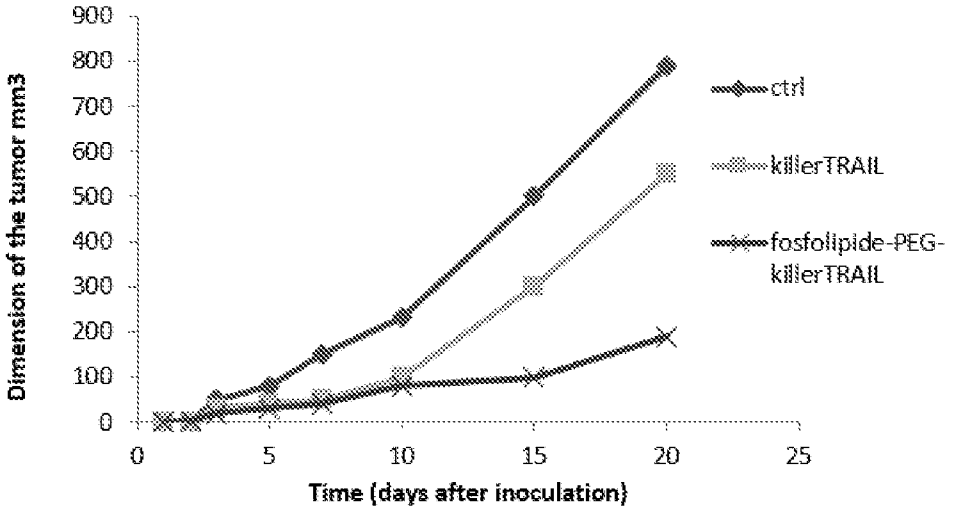

Figure 13

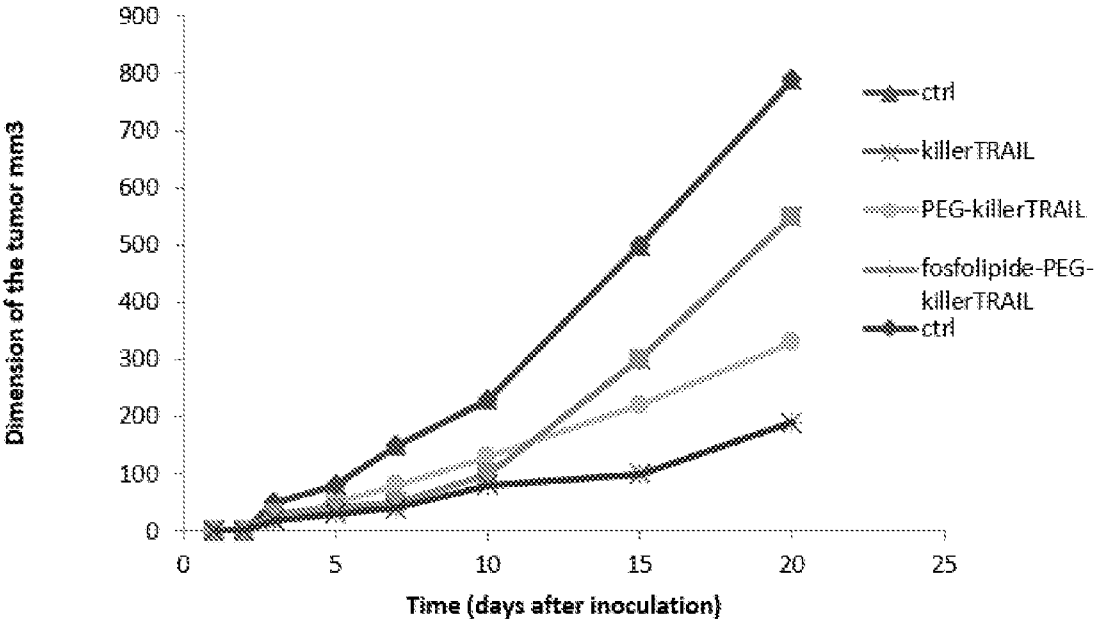

Figure 14

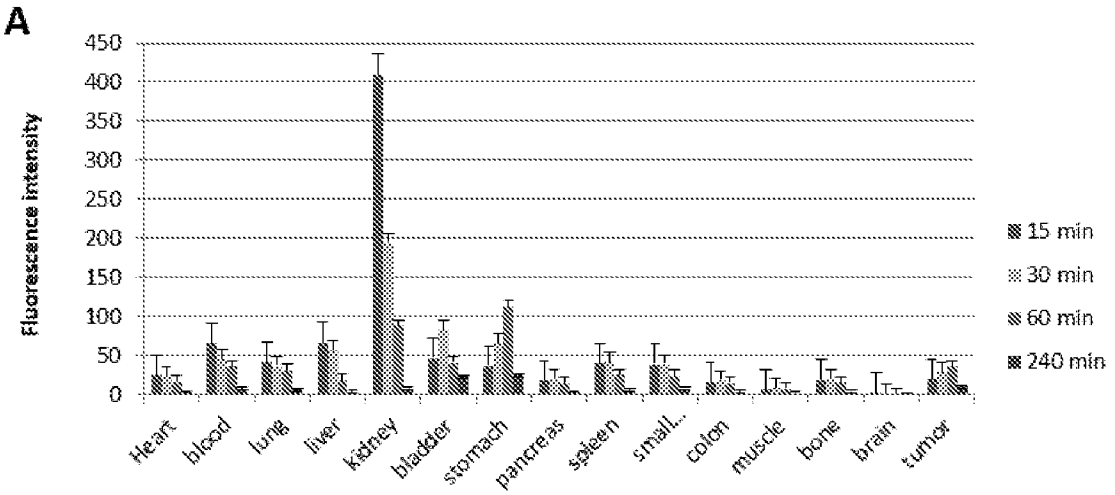

Figure 15A

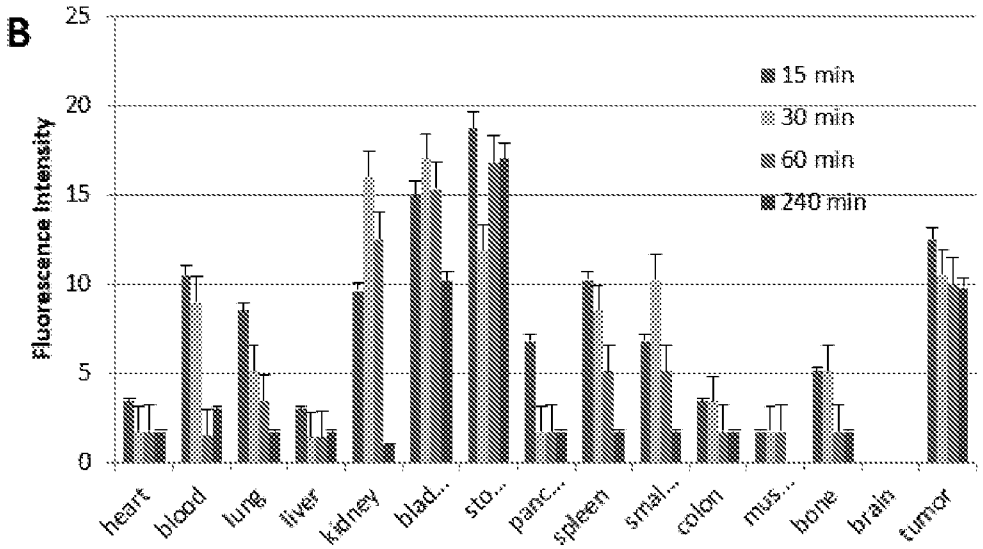

Figure 15B

C

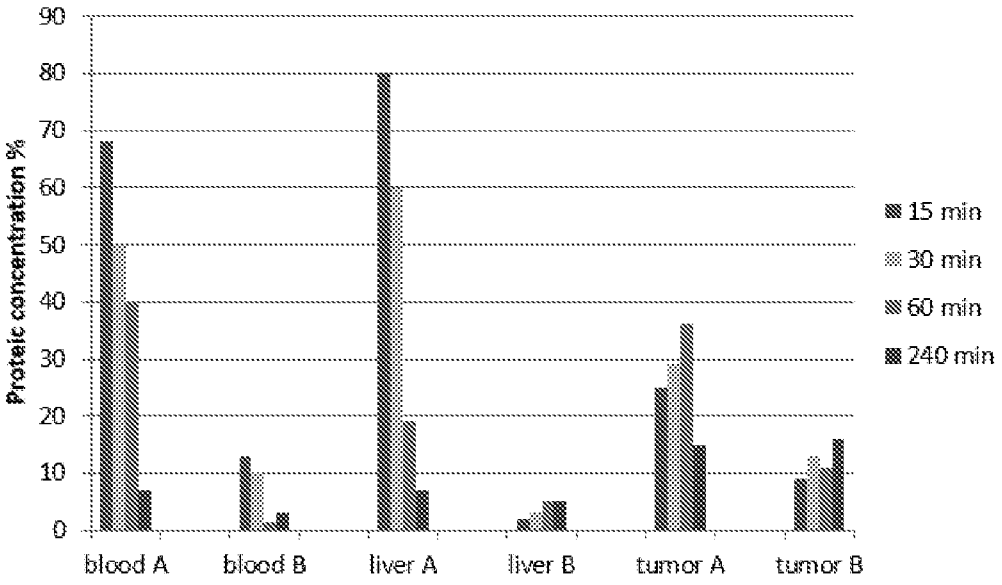
90
80
70
60
50
40
30
20
10
0
Protein concentration %
blood A
blood B
liver A
liver B
tumor A
tumor B
15 min
30 min
60 min
240 min

Figure 15C

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/054787

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/69 A61K47/62 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OLIVER SEIFERT ET AL: "Immuno-LipoTRAIL: Targeted Delivery of TRAIL-Functionalized Liposomal Nanoparticles", BIOCONJUGATE CHEMISTRY, vol. 25, no. 5, 7 May 2014 (2014-05-07), pages 879-887, XP055751958, ISSN: 1043-1802, DOI: 10.1021/bc400517j	1-14, 18-40
Y	Figure 2,6; page 880-882; page 884, left column; page 885, right column, bottom paragraph; page 886, left column, top paragraph and sections "Pharmacokinetics" and "Tumor Models"	15-17,41

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 September 2021	23/09/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Burema, Shiri

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/054787

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Oliver Seifert: "TRAIL-based multivalent and multifunctional fusion proteins and liposomes for therapeutic applications", , 1 January 2014 (2014-01-01), XP055207991, Retrieved from the Internet: URL:http://elib.uni-stuttgart.de/opus/volltexte/2014/9280/pdf/Dissertation_Oliver_Seifert.pdf [retrieved on 2015-08-17] paragraph 2.6 and subparagraphs (pages 51-54); paragraph 3.2 and subparagraphs (pages 95-107); paragraph 4.3 (pages 116-119)	1,3,4, 8-13, 18-29, 31-36, 38,39
X	LOI M ET AL: "sTRAIL coupled to liposomes improves its pharmacokinetic profile and overcomes neuroblastoma tumour resistance in combination with Bortezomib", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 192, 17 July 2014 (2014-07-17), pages 157-166, XP029060804, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2014.07.009 Paragraphs 2.1, 2.2, 2.4, 2.5, 2.7, 2.8, 3.3, 3.4, 3.7; page 158, last paragraph of left column, first paragraph of right column; table 1	1,3,4, 8-13, 18-29, 31-36, 38,39
X	PRADEEP M. NAIR ET AL: "Enhancing the antitumor efficacy of a cell-surface death ligand by covalent membrane display", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 112, no. 18, 20 April 2015 (2015-04-20), pages 5679-5684, XP055751968, US ISSN: 0027-8424, DOI: 10.1073/pnas.1418962112 Supporting information: page 1, paragraph "Formulating liposomes for Membrane Display of Apo2L/Trail"; page 2, paragraph "Animal Studies Using Apo2LL" and "Fluorescence imaging of Apo2L.L"	1,3,4, 8-13, 18-29, 31-35

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	-& PRADEEP M. NAIR ET AL: "Supporting Information: Enhancing the antitumor efficacy of a cell-surface death ligand by covalent membrane display", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 112, no. 18, 20 April 2015 (2015-04-20), pages 5679-5684, XP055751970, US ISSN: 0027-8424, DOI: 10.1073/pnas.1418962112 page 1, paragraph "Formulating liposomes for Membrane Display of Apo2L/Trail"; page 2, paragraph "Animal Studies Using Apo2LL" and "Fluorescence imaging of Apo2L.L"	
A	- Peprotech: "Recombinant Human sTRAIL/Apo2L", , 1 January 2020 (2020-01-01), XP055752000, Retrieved from the Internet: URL:https://www.peprotech.com/en/recombinant-human-strailapo2l [retrieved on 2020-11-18] the whole document	1-41
A	- Genscript: "TRAIL/Apo2L, Human", , 1 January 2020 (2020-01-01), XP055752237, Retrieved from the Internet: URL:https://www.genscript.com/protein/Z03124-TRAIL_Apo2L_Human.html [retrieved on 2020-11-19] the whole document	1-41
A	- Enzo Life Sciences: "KillerTRAIL(TM) Protein (soluble) (human), (recombinant) - ALX-201-073 - Enzo Life Sciences", , 1 January 2020 (2020-01-01), XP055751955, Retrieved from the Internet: URL:https://www.enzolifesciences.com/ALX-201-073/killertrail-protein-soluble-human-recombinant/ [retrieved on 2020-11-18] the whole document	1-41

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/054787

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DIEGO DE MIGUEL ET AL: "Double-Edged Lipid Nanoparticles Combining Liposome-Bound TRAIL and Encapsulated Doxorubicin Showing an Extraordinary Synergistic Pro-Apoptotic Potential", CANCERS, vol. 11, no. 12, 5 December 2019 (2019-12-05), page 1948, XP055681277, DOI: 10.3390/cancers11121948 Abstract, Figure 1	15-17,41
Y	JIANG TIANYUE ET AL: "Gel-Liposome-Mediated Co-Delivery of Anticancer Membrane-Associated Proteins and Small-Molecule Drugs for Enhanced Therapeutic Efficacy", ADVANCED FUNCTIONAL MATERIALS, vol. 24, no. 16, 2 January 2014 (2014-01-02), pages 2295-2304, XP055775940, DE ISSN: 1616-301X, DOI: 10.1002/adfm.201303222 Abstract, Figure 1	15-17,41
Y	HUANG SHIQI ET AL: "Improved melanoma suppression with target-delivered TRAIL and Paclitaxel by a multifunctional nanocarrier", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 325, 3 April 2020 (2020-04-03), pages 10-24, XP086251675, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2020.03.049 [retrieved on 2020-04-03] Abstract and scheme 1	15-17,41
Y	EP 2 177 230 A1 (DAIICHI SANKYO CO LTD [JP]) 21 April 2010 (2010-04-21) paragraphs [0231] and [0310]	15-17,41
A	EP 2 468 260 A2 (UNIV ZARAGOZA [ES]; UNIV PAIS VASCO [ES] ET AL.) 27 June 2012 (2012-06-27) claim 1, SEQ ID NO: 2.	1-41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/054787

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2021/054787

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2177230 A1	21-04-2010	CA 2695991 A1	12-02-2009
		CN 101820913 A	01-09-2010
		EP 2177230 A1	21-04-2010
		EP 2184355 A1	12-05-2010
		JP WO2009020093 A1	04-11-2010
		JP WO2009020094 A1	04-11-2010
		KR 20100046185 A	06-05-2010
		TW 200914064 A	01-04-2009
		TW 200916477 A	16-04-2009
		US 2010209490 A1	19-08-2010
		US 2011269942 A1	03-11-2011
		WO 2009020093 A1	12-02-2009
		WO 2009020094 A1	12-02-2009
EP 2468260 A2	27-06-2012	EP 2468260 A2	27-06-2012
		ES 2356880 A1	14-04-2011
		US 2012189690 A1	26-07-2012
		WO 2011020933 A2	24-02-2011