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(71) Applicants: SOCIETE DE COMMERCIALISATION DES PRODUITS DE LA RECHERCHE APPLIQUEE SOCPRA SANTE ET HUMAINES, S.E.C. [CA/CA]; A/ S TransferTech Sherbrooke, 35 rue Radisson, Suite 200, Sherbrooke, Québec J1L 1E2 (CA). OVENS INNOVATIONS INC. [CA/CA]; 500 boulevard Cartier Ouest, Laval, Québec H7V 5B7 (CA).

(72) Inventors: GOBEIL, Fernand Jr.; 2070 rue du Vermont, Sherbrooke, Québec J1H 3P4 (CA). DORY, Yves; 636 route 210, Cookshire-Eaton, Québec J0B 1M0 (CA). SAVARD, Martin; 336 rue des Trembles, Sherbrooke, Québec J1G 4T9 (CA). GAGNON, Maxime; 205-2970 rue du Sauvignon, Sherbrooke, Québec J1L 0A2 (CA). TREMBLAY, Philippe; 21 rue du Camarguais, Blainville, Québec J7C 4P4 (CA). MOQUIN, Alexandre; 3766 rue de Mentana, Montréal, Québec H2L 3R3 (CA). GAGNE, Stéphane; 446-3000 12e Avenue Nord, Sherbrooke, Québec J1H 5H3 (CA).

(74) Agent: NORTON ROSE FULBRIGHT CANADA S.E.N.C.R.L., S.R.L. / LLP; 2500-1 Place Ville-Marie, Montreal, Québec H3B 1R1 (CA).

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(54) Title: NANOPARTICLES COMPRISING N,N,N-TRIMETHYL CHITOSAN COATED WITH PEPTIDE AGONISTS OF KININ B1 OR B2 AND/OR PEPTIDE ANTAGONISTS OF KININ B1 FOR TRANSVASCULAR DRUG DELIVERY ACROSS THE BLOOD-BRAIN BARRIER

(57) Abstract: It is provided a nanoformulation comprising N,N,N-trimethyl chitosan (TMC), an agonist or an antagonist of kinin B2 or B1 receptor and a payload to be delivered across the blood-brain barrier (BBB). The nanoformulation can be used to treat central nervous system (CNS) by for example an intravenous (IV) or intranasal (IN) administration.

- 1 -

NANOPARTICLES COMPRISING N,N,N-TRIMETHYL CHITOSAN COATED WITH PEPTIDE AGONISTS OF KININ B1 OR B2 AND/OR PEPTIDE ANTAGONISTS OF KININ B1 FOR TRANSVASCULAR DRUG DELIVERY ACROSS THE BLOOD-BRAIN BARRIER

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application is claiming priority from U.S. Provisional Application No. 63/508,938 filed June 19, 2023, the content of which is hereby incorporated by reference in their entirety.

TECHNICAL FIELD

[0002] It is provided a nanoformulation comprising N,N,N-trimethyl chitosan (TMC), an agonist or an antagonist of kinin B2 or B1 receptor and a payload to be delivered across the blood-brain barrier (BBB).

BACKGROUND

[0003] Alzheimer's disease (AD) is a progressive neurodegenerative disorder affecting elderly people worldwide and is mainly characterized by two major pathological hall-marks in the AD brain: extracellular plaques, containing various forms of amyloid- β protein ($A\beta$), and intracellular neurofibrillary tangles (NFTs), composed of hyper-phosphorylated tau protein. However, the recent failures in drug development along with the only available symptomatic drugs currently on the market for AD that have no effect on disease progression leave a large unmet medical need. As such, there is a consensus among the community for the need to discover new, more efficient therapeutic targets in a multi-combination approach that ultimately aim to stop or reverse early.

[0004] AD immunotherapeutic drugs, such as monoclonal antibodies (mAbs), have been developed rapidly in the last decade, but major challenges of these immunotherapeutic approaches are poor blood-brain barrier (BBB) penetration, induction of inflammation and microhemorrhages, absence of significant cognitive effects, and off-target reactivity.

[0005] The blood-brain barrier (BBB) is a major obstacle to the development of effective therapeutics for Alzheimer's disease (AD) and other central nervous system (CNS) disorders. Specifically, low CNS penetration or complete prevention of delivery across the BBB has precluded the advancement of countless molecules with therapeutic potential. Antibody therapeutics have been particularly challenging due to their large size; however, their target specificity, reduced off-target effects and prolonged pharmacokinetics make them a very attractive treatment strategy. To date, many

- 2 -

avenues have been explored to overcome the challenge offered by the BBB. However, none of these approaches has demonstrated the adequate balance of robustness, safety, and desirable physicochemical properties necessary for an effective brain drug delivery system.

[0006] It is thus highly desired to be provided with means to allow drugs CNS penetration and/or delivery across the BBB.

SUMMARY

[0007] It is provided a nanoformulation comprising N,N,N-trimethyl chitosan (TMC); a peptide agonist of kinin B1 receptor, a peptide antagonist of kinin B1 receptor or a peptide agonist of kinin B2 receptor; and a payload to be delivered across the blood-brain barrier (BBB).

[0008] In an embodiment, the agonist of kinin B1 receptor, the antagonist of kinin B1 receptor or the agonist of kinin B2 receptor is coupled to the TMC.

[0009] In another embodiment, the nanoformulation described herein comprises a spacer coupling the agonist of kinin B1 receptor, the antagonist of kinin B1 receptor or the agonist of kinin B2 receptor to the TMC.

[0010] In an embodiment, the spacer is a Mpa, Mpa- β Ala spacer, Mpa-Aca, Mpa-PEG2, Mpa-PEG8, or Mpa-Gly.

[0011] In another embodiment, the nanoformulation encompassed herein comprises a ratio of 1: 7 of TMC: B1R agonist or antagonist.

[0012] In another embodiment, the nanoformulation encompassed herein comprises a B1R agonist coupled to TMC and a non-cross-linked B2R agonist.

[0013] In a further embodiment, the nanoformulation encompassed herein comprises a non-cross-linked B1R agonist and a non-cross-linked B2R agonist.

[0014] In another embodiment, the nanoformulation encompassed herein comprises a B1R antagonist coupled to TMC and a non-cross-linked B2R agonist.

[0015] In a further embodiment, the nanoformulation encompassed herein comprises a non-cross-linked B1R antagonist and a non-cross-linked B2R agonist.

- 3 -

[0016] In a particular embodiment, the nanoformulation encompassed herein comprises at least one B1R agonist selected from the group consisting of:

β Ala-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

β Ala-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Gly-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Gly-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Sar-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Sar-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Aca-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Aca-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; and

Lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH.

[0017] In a particular embodiment, the nanoformulation encompassed herein comprises a B1R consisting of β Ala-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH; β Ala-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; Gly-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH; Gly-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; Sar-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH; Sar-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; Aca-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH; Aca-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; or Lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH.

[0018] In another embodiment, the nanoformulation encompassed herein comprises at least one B2R agonist selected from the group consisting of:

Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH;

Lys-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

β Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH; and

- 4 -

Aca-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH.

[0019] In another embodiment, the nanoformulation encompassed herein comprises a B2R consisting of Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH; Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH; Lys-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH; β Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH; or Aca-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH.

[0020] In another embodiment, the nanoformulation encompassed herein comprises at least one B1R antagonist selected from the group consisting of:

AcOrn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

AcLys-Lys-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

Aca-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; and

Lys-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH.

[0021] In another embodiment, the nanoformulation encompassed herein comprises a B1R antagonist consisting of AcOrn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; AcLys-Lys-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; Aca-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; or Lys-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH.

[0022] In a further embodiment, the agonist or antagonist of kinin B1 receptor further comprises a chelating compound at the N-terminus.

[0023] In an embodiment, the chelating compound is 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA); 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA); methylhydroxamates derived from triaza- and tetraaza macrocycles (NOTHA2 and DOTH2); 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA), or a derivative thereof.

- 5 -

[0024] In another embodiment, the nanoformulation encompassed herein comprises at least one B1R agonist, B2R agonist, or B1R antagonist selected from the group consisting of:

Mpa- β Ala-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Mpa- β Ala-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Mpa-Gly-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Mpa-Gly-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Mpa-Aca-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Mpa-Aca-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

H-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

H-Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH;

Mpa- β Ala-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

H-Lys(Mpa)-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

Mpa- β Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH;

Mpa-Aca-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

Mpa- β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

Mpa-PEG2- β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

AcLys(Mpa)-Lys-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

Mpa-Aca-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; and

NOTA-Aca-Lys(Mpa)-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH.

- 6 -

[0025] In another embodiment, the nanoformulation encompassed herein comprises a ratio of 2:1 of agonist or an antagonist B1R ligand: agonist of B2R.

[0026] In an embodiment, the agonist of kinin B1 receptor, the antagonist of kinin B1 receptor or the agonist of kinin B2 receptor further comprises a radiolabeling agent at its N-terminus.

[0027] In an embodiment, the radiolabeling agent is ^{64}Cu , ^{67}Cu , ^{68}Ga , ^{131}I , ^{111}In , ^{153}Sm , ^{89}Sr , ^{90}Y , ^{177}Lu , ^{225}Ac or ^{213}Bi .

[0028] In a further embodiment, the payload is siRNA, a shRNA, an antibody, a small molecule, an antisense, a polynucleotide, or an aptamer.

[0029] In another embodiment, the antibody is a monoclonal antibody, a polyclonal antibody or a humanized antibody.

[0030] In an embodiment, the nanoformulation encompassed herein is formulated for an intravenous (IV) administration or intranasal (IN) administration.

[0031] It is encompassed other routes of administration, such as and not limited to, intraarterial administration and convection-enhanced delivery (CED).

[0032] In a further embodiment, the nanoformulation encompassed herein is for the treatment of a central nervous system (CNS) disease.

[0033] In a further embodiment, the nanoformulation encompassed herein is for measuring the biological effects of the payload to treat a CNS disorder.

[0034] It is also provided a method of treating a CNS disorder comprising the step of administering the nanoformulation encompassed herein.

[0035] It is also provided the use of the nanoformulation encompassed herein for treating a CNS disorder. In addition, it is encompassed the use of the nanoformulation described herein in the manufacture of a medication for treating a CNS disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] Reference will now be made to the accompanying drawings.

[0037] Fig. 1 illustrates the chemical synthesis of kinin agonists functionalized TRIOZAN™ NPs.

- 7 -

[0038] Fig. 2 illustrates representative curves showing displacements of [³H]-Kallidin-[DesArg¹⁰-Leu⁹] (A, C) and [³H]-BK (B) binding to human recombinant B1R and B2R, respectively, by the mAbs-unloaded F4 (A, B) and F7 (C) formulations. Displacement curves were fitted using a one-site model.

[0039] Fig. 3 illustrates quantification of mAbs by organ-to-serum ratio in organ tissues ((ng/g of wet tissue)/(ng/mL of serum)) following intravenous injection of TRIOZANTM nanoformulations in AD transgenic mice (males and females combined) at 24h post-delivery. Data are presented as mean ± SEM, F1: n=9, F4/F6: n=6, *** p<0.001, **** p<0.0001 (multiple comparisons using two-way ANOVA with Sidak's correction).

[0040] Fig. 4 illustrates quantification of mAbs in organ tissues (ng/mg of wet tissue) following intranasal administration of TRIOZANTM nanoformulations in AD transgenic mice (males and females combined) at 24h post-delivery. Data are presented as mean ± SEM, n=6, ** p<0.001, **** p<0.0001 (multiple comparisons using two-way ANOVA with Sidak's correction).

[0041] Fig. 5 illustrates comparison of tissue biodistribution of free mAb (F1) and mAb loaded-TRIOZANTM NPs containing both B1R/B2R agonists (F4 and F6) following intravenous and intranasal administrations in AD transgenic mice (males and females combined) at 24h post-delivery. Calculated IV/IN ratios ((ng mAbs/mg tissue) / (ng mAbs/mg tissue)) determined from n= 6-9 per group. Data represent mean ± SEM.

DETAILED DESCRIPTION

[0042] In accordance with the present description, it is provided a nanoformulation comprising N,N,N-trimethyl chitosan (TMC), an agonist or an antagonist of kinin B2 or B1 receptor and a payload to be delivered across the blood-brain barrier (BBB).

[0043] Therefore the provided kinin-TRIOZANTM nanoparticles (NPs) allow mediated targeted delivery of drugs for the treatment of CNS diseases, and for elucidating and optimizing biological effects of novel drug candidates to treat CNS disorders.

[0044] An emerging and promising field for drug delivery across the BBB involves nanocarrier-based systems. Among all drug delivery nanosystems currently undergoing active research and approved for biomedical applications, there are the chitosan- and chitosan derivative-based nanocarriers. Prominent among the latter is TRIOZANTM, which uses a single hydrophilic quaternized biopolymer N,N,N-trimethyl chitosan (TMC).

- 8 -

The biobased, self-assembled TMC nanocarriers allow efficient encapsulation and protection of drug molecules against degradation while simultaneously maintaining therapeutic integrity. TMC has been shown to be readily soluble in water (over a wide range of pH), safe, biodegradable, biocompatible, and can be easily chemically conjugated for active targeting of the loaded therapeutic agent. Moreover, because of its good mucoadhesive properties, it has been used to develop nanocarriers for brain targeting via intranasal administration, additionally to standard intravenous injections.

[0045] Yet, another strategy to enhance drug delivery to the brain involves the targeted permeabilization of the BBB at inflammatory sites through the activation of overexpressed G proteins-coupled receptors (GPCRs), and that includes the kinin B2 and B1 receptors (B2R and B1R). Indeed, metabolically resistant, B1R or B2R selective kinin peptide agonists have been shown effective in improving the delivery of drugs into the CNS via transient opening of the BBB with no significant side effects such as brain edema. Moreover, a dual B1R/B2R agonist has been used to modulate the BBB paracellular permeability (Côté et al., 2013, *Cancer Biol Ther*, 14: 806-811).

[0046] Herein it is provided a new type of nanodelivery system based on kinin-B1R/B2R agonists coated nanoparticles (NPs) to promote effective transvascular drug delivery across the pathological BBB, which leads to increased brain penetration and therapeutic potency of a drug or payload that needs to cross the BBB, such as a siRNA, a shRNA, an antibody, a small molecule, an antisense, a polynucleotide, or an aptamer. In an embodiment, the antibody is a monoclonal antibody, a polyclonal antibody or a humanized antibody. The relative biodistribution profiles of a mAb loaded into these NPs versus the naked mAb were assessed *in vivo* through two routes of administrations (intravenous (IV), intranasal (IN)) in the Tg-SwDI mouse model of AD.

[0047] It is encompassed other routes of administration, such as and not limited to, intraarterial and convection-enhanced delivery (CED).

[0048] Chemical synthesis of kinin agonists functionalized TRIOZAN™ NPs was accomplished in the following way (Fig. 1). First the agonist sequence was prepared on solid support using standard SPPS procedures (Fmoc strategy, step 1). In the same way the two-residues Mpa-βAla spacer was introduced (step 2). In a third step the full spacer-agonist peptide was released off the resin as its free thiol. Finally (step 4), the free thiol was coupled at the 6-OH position of TRIOZAN™ subunits that had been freshly activated as its corresponding tosylate (S_N2 reaction leading to thioethers), thus yielding the

- 9 -

desired TRIOZAN™ polymers. According to the number of equivalents of peptide free thiol used during step 4, the degree of conjugation varies.

[0049] It is encompassed that the spacer can be e.g. Mpa-βAla, Mpa-Aca, Mpa-PEG2, Mpa-PEG8, or Mpa-Gly.

[0050] The results provided herewith show that the conjugation of TRIOZAN™ to the N-termini of biostable peptide B1R agonist (NG29) and antagonist (R954) covalently extended with Mpa/βAla spacer, is well tolerated, providing TMC-peptide conjugates with a ratio 1:7 having high affinities towards the human B1R (hB1R, Table 1). By comparison, TMC-B1R agonist conjugates prepared with a 1:1 stoichiometry led to a ~400-fold reduction of the binding affinity toward hB1R (IC₅₀ value: 200 nM) compared to the parent unmodified peptide agonist, hence, this option was discarded. When applied to the biostable B2R agonist NG291, the N-terminal extension with Mpa was found to have detrimental effects on its binding capacity to hB2R (MAB7053 (IC₅₀ value: 1,180 nM) vs. NG291 (IC₅₀ value: 2 nM); Table 1). Such negative effects were also observed with the use of other types of linkers (e.g., PEG2, PEG8, Gly). Moreover, the bioconjugation procedure of TMC and N-terminal Mpa-NG291 with the optimal ratio 1:7 found for the B1R agonist coupling to TMC led to a further decrease of the resulted TMC-B2R conjugate for its B2R target (TRIOZAN™-MAB7053 IC₅₀ value: 10,600 nM, Table 1). To circumvent the possible steric hindrance effect of the linker affecting NG291-B2R interaction, the strategy of creating B1R functionalized TRIOZAN™-NPs complexed with free (non-cross-linked) B2R agonists for delivering the mAbs across the BBB (nanoformulation F4) was adopted. Another version of dual B1R/B2R peptides NPs comprising a mix 2:1 of unconjugated B1R and B2R agonists was prepared to possibly maximize both ligand biological activity (nanoformulation F6).

Table 1: Binding affinities of novel peptide conjugates on HEK293 cells stably overexpressing human kinin B1 and B2 receptors

Sequence (codename)	Theoretical m.w.	Observed m.w	Purity (%)	B1R-HEK293 IC ₅₀ (nM)
H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH (LysdesArg ⁹ BK)	1032.2	1032.4	99.4	0.7
Sar-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH (NG29)	1103.3	1103.5	99.1	0.5

- 10 -

Mpa-βAla-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH (MAB7108)	1191.4	1191.9	100	0.8
TRIOZAN™-MAB7108 (7-Eq)	73 217	-	-	0.4
TRIOZAN™-MAB7108 (1-Eq)	66 174	-	-	193
Mpa-βAla-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH (ST1002)	1233.6	1233.6	100	0.3
TRIOZAN™-ST1002 (ST1119)	58 647	-	-	3.3
NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH (ST1116)	1688.3	1689.8	96.4	11
NOTA-Aca-Lys(Mpa)-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH (ST1117)	1560.1	1561.7	100	176

Table 2: Binding affinities of novel peptide B1R antagonist conjugates on HEK293 cells stably expressing human kinin B1 receptors

Sequence (codename)	Theoretical m.w.	Observed m.w	Purity (%)	B1R-HEK293 IC50 (nM)
AcOrn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-dβNal-Ile-OH (R954)	1194.4	1194.5	99.2	2
Mpa-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-dβNal-Ile-OH (AG1008/MAB7070/MAB7122)	1311.6	1312.1	99.8	13
Mpa-PEG2-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-dβNal-Ile-OH (MAB7123)	1456.8	1457.3	99.9	23
Mpa-PEG8-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-dβNal-Ile-OH (AGT7123)	1735.1	1735.2	96.7	72
TRIOZAN™-MAB7122 (7-Eq)	74 059	-	-	18
TRIOZAN™-MAB7123 (7-Eq)	75 082	-	-	55
TRIOZAN™-MAB7070 (7-Eq/ DQ: 45%) (ST1109)	74 177	-	-	8
TRIOZAN™-MAB7070 (7-Eq/ DQ: 60%) (ST1110)	59 177	-	-	31
Ac-Lys(Mpa)-Lys-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-dβNal-Ile-OH (AGT0382-2)	1424.8	1425.0	99.4	8

- 11 -

Mpa-Aca-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser- β Nal-Ile-OH (ST1001)	1353.7	1353.8	99.0	31
NOTA-Aca-Lys(Mpa)-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser- β Nal-Ile-OH (ST1003)	1765.2	1767.1	99.8	14

Table 3: Binding affinities of novel peptide B2R agonist conjugates on HEK293 cells stably expressing human kinin B2 receptors

Sequence (codename)	Theoretical m.w.	Observed m.w	Purity (%)	B2R-HEK293 IC50 (nM)
H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (BK)	1060.2	1060.4	99.0	4
H-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (NG291)	1130.4	1130.5	100	2
Mpa- β Ala-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (MAB7053)	1289.6	1289.8	100	1 180
Mpa-PEG2-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (MAB7114)	1434.7	1435.1	99.4	1 739
Mpa-PEG8-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (MAB7137)	1642.0	1642.2	99.7	3 120
Mpa-PEG8-Lys-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (MAB7138)	1770.1	1770.4	100	10 500
H-Lys(Mpa)-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (AGT29OCT65)	1346.6	1346.8	100	960
H-Cys-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (AGTB2R-1)	1220.5	1220.7	98.3	1 160
Mpa- β Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (AGTB2R-2)	1219.4	1219.7	98.3	1 175
TRIOZAN TM -MAB7053 (7-Eq)	73 910	-	-	10 600
Mpa-Aca-Arg-Pro-Hyp-Gly-Thi-Ser- ^N Chg-Thi-Arg-OH (AGT0382)	1331.6	1331.9	98.8	1 041
H-Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH (ST1100)	1220.3	1220.8	99.9	182
H-Lys-Arg-Pro-Hyp-Gly-Phe-Lys(Mpa)-Pro-Phe-Arg-OH (ST1115)	1333.2	1334.5	100	> 10 000

Table 4: Binding affinities of kinin analogues linked to or complexed with TRIOZAN™ NPs on HEK293 cells stably expressing hB1R or hB2R

Compound codenames /stock concentration	DLS (nm)	PDI	hB1R IC50 (µg/ml)	hB2R IC50 (µg/ml)
MAB7108 coupled to TRIOZAN™-NP (1 mg/mL)	60	0.312	1.13	n.d.
NG291 complexed with TRIOZAN™-NP (3 mg/mL)	51	0.301	n.d.	1.46
MAB7108 coupled, NG291 complexed TRIOZAN™-NP (1 mg/mL)	59	0.290	3.56	1.53
MAB7122 coupled to TRIOZAN™-NP (1 mg/mL)	78	0.251	7.12	n.d.

[0051] It is further encompassed that the B1R agonist, B1R antagonist or B2R agonist peptides are:

B1R agonist peptides:

Sar-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH
Mpa-βAla-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH
Mpa-βAla-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH
Mpa-Gly-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH
Mpa-Gly-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH
Mpa-Aca-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH
Mpa-Aca-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH
*NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH
*NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH
* For radiolabeling with ^{64/67}Cu and use for PET imaging of B1R and theranostics

B2R agonist peptides:

H-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH
H-Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH
Mpa-βAla-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH
H-Lys(Mpa)-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH
Mpa-βAla-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
Mpa-Aca-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH

B1R antagonist peptides:

AcOrn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH
Mpa-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH
Mpa-PEG2-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH
AcLys(Mpa)-Lys-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH
Mpa-Aca-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH
*NOTA-Aca-Lys(Mpa)-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH

* For radiolabeling with $^{64/67}\text{Cu}$ and use for PET imaging of B1R and theranostics

[0052] TRIOZANTM is a positively charged polymer owing to its numerous quaternary ammonium groups. It can be electrostatically cross-linked by adding negatively charged polytriphosphate (3PP) leading to the formation of stable NPs, that act as nanocontainers. As an example of a payload, but not limited to, is a mAb, which is non-covalently retained inside the NPs until they reach the desired location for delivery. This selectivity is provided by B1R and B2R ligands. Similar to the mAb, these ligands can be non-covalently trapped inside the NPs, from which they can slowly leach out to bind to the cognate receptors. This corresponds to nanoformulation F6, for which the NPs contain twice as much B1R ligand as B2R ligand. This was done to limit potential hypotensive effects of NPs containing NG291 agonists, mostly attributable to systemic vascular B2R activation. Alternatively, the ligands can be covalently attached to the NPs by a spacer, meaning that binding between receptors and ligands can be somehow hindered by the bulk of the NPs. Moreover, ligands trapped inside the NPs are in no position to meet their receptors counterparts. Therefore, a loss of affinity is to be expected in that situation as indeed observed in nanoformulation F4, where the B1R ligand only is covalently linked to the NP, whereas the B2R ligand is not.

[0053] Binding experiments with HEK-293 cells stably expressing human B1R (Fig. 2, panel A) or B2R (Fig. 2, panel B) were also performed with new unloaded, peptide B1R agonists functionalized TRIOZANTM-NPs mixed with B2R agonists. Results showed that the NPs, prepared using the specified starting material described in Table 1 (below referred to as F4 nanoformulation, see Table 5), retained their abilities to bind to human B1R 186 and B2R, and being able to fully displace selective radioligands.

[0054] The mAb liquid solution (F1; 2.5 mg/mL) and the new TRIOZANTM-NP formulations loaded with mAb, F4 and F6, in lyophilized forms (in PBS/sucrose) were readily soluble in deionized water. The chosen 2.5 mg/mL of F1 corresponds to a dose of 10 mg/kg by injecting a volume of 4 mL/kg (e.g. 80 μL for a 20 g mouse). A summary analysis of the characteristics of the mAb solution and the water-soluble NP formulations, is presented in Table 5. The final concentrations of loaded mAb in F4 and F6 NPs were in the same range as the original mAb solution (2.5-3.3 mg/mL). The mean diameters of NPs were found to be less than 200 nm with polydispersity index (PDI) values below 0.3, indicating monodisperse NP suspensions. Due to their favorable characteristics

- 14 -

(aqueous solubility, B1R/B2R binding capacity, uniform and small sizes (i.e., between 130 and 165 nm)), the new NPs were considered appropriate for in vivo studies.

Table 5: Comparative analysis of the physicochemical characteristics of new TRIOZAN™-NPs

Formulations	[mAb] (mg/ml)	Z-average (nm)	PDI
F1: mAb	2.74 ± 0.09	11.3 ± 0.2	0.020 ± 0.010
F4: TMC/TMC-B1Rago-DS-TPP (mAb + B2R ago)	3.25 ± 0.04	165.6 ± 5.5	0.213 ± 0.004
F6: TMC-DS-TPP (mAb + B1R ago + B2R ago)	2.97 ± 0.11	132.4 ± 2.3	0.144 ± 0.006
F7: TMS/TMC-B1Rantago-DS-TPP (mAb)	3.27 ± 0.10	189.7 ± 3.1	0.231 ± 0.016

Date are means ± SD. F1: Test mAbs (2.5 mg/mL); F4 TMC/TMC-B1R ago: dextran sulfate (DS): tripolyphosphate (TPP) (5.4/0.6: 1: 0.5 in wt: wt: wt): mAb 2.5 mg/mL + B2R ago 45 µM; and F6: TMC: DS: TPP (6: 1: 0.5): mAb 2.5 mg/mL + B1R ago 90 µM + B2R ago 45 µM; F7: TMC/TMC-B1R antago: DS: TPP (5.4/0.6: 1: 0.5 in wt: wt: wt): mAb 2.5 mg/mL.

[0055] Prior to performing the mAb tissue biodistribution experiments, an acute, single-dose toxicity assessment of unloaded B1R/B2R targeted-NPs (F4 and F6) was carried out in conscious adult normal mice. No sign of abnormal behavior or distress was observed upon their intravenous (IV) tail vein injection during a period of 24h.

[0056] Biodistribution studies were then performed to characterize the relative effects of the two NP preparations on the accumulation of the tested mAb at specific regions of the brain such as, entorhinal cortex and hippocampus (regions responsible for learning and memory), most likely to be affected in AD. Comparison of biodistribution profiles of the free mAb and as NPs was evaluated at 24h post-injection. For the purpose of these experiments, a new, highly sensitive LC-MS/MS assay was developed for a direct quantification of the tested mAb in blood and tissue extracts (specifically, brain, liver, serum) from transgenic AD adult mice. The naked mAb (F1) and the new NPs formulations containing the mAb encapsulated with the B2R agonist in B1R-agonist-conjugated TRIOZAN™-NPs (F4), or the mAb encapsulated with the B1R and B2R agonists inside TRIOZAN™-NPs (F6), were administered by IV injections and organ-to-serum ratios of mAb were quantified (Fig. 3). IV injections of F4 and F6 led to ~4- and 12-fold increases, respectively, in brain delivery of mAb compared to F1 in AD mice, indicating cerebral bioavailability enhancement by these two formulations. Furthermore, there was no apparent sex difference in the brain uptake of the tested formulations given IV. Looking specifically at F6, the amounts of mAb found in the brains were about 60-fold higher than that in livers. As seen in Fig. 3, liver uptakes remained relatively low with similar values for the two tested formulations. Interestingly, despite the final

- 15 -

concentration of B1R agonist being the same between F4 and F6 (i.e., 90 μ M), the latter resulted in significantly more mAb accumulation in both hippocampus and the entorhinal cortex regions (Fig. 3). This suggests that the complexation rather than the covalent coupling of B1R agonists to TRIOZAN™ is more suited for increasing the permeability of the BBB by the B1R. Such inference is supported by the binding data showing a reduced binding affinity of the B1R agonist (~15-fold lower) when tethered onto TRIOZAN™ as compared to the unmodified agonist.

[0057] The intranasal (IN) administration route has recently gained increasing interest for delivering drugs to the CNS while bypassing the BBB. Therefore, for comparison purposes, the potential of the new drug-loaded nanoformulations was investigated to distribute into the brain after IN administration. For this, the naked mAb alone (F1) and the mAb loaded-NP formulations (F4 and F6) were administered by intranasal drops at the same mAb dosage of 10 mg/kg. The nanoformulations were well-tolerated with no apparent adverse effects for up to 24h after their administration. At 24h post-delivery, significant increases in the mAb levels were observed in the entorhinal cortices and hippocampi of mice treated with the F4 (3- to 4-fold) and F6 (4-to 5-fold) versus F1 (Fig. 4). Systemic exposure was low and highly variable for all formulations upon their IN administrations.

[0058] A comparison of the brain biodistribution of the mAb contained in F1, F4, and F6, following IV and IN administrations in AD transgenic mice revealed that the systemic IV mode of administration is highly superior to IN for the targeted delivery of both free mAbs and the mAbs-loaded TRIOZAN™ NPs at AD brain regions (ranging from ~5- to 15-fold higher) (Fig. 5).

[0059] Altogether, it is provided the efficacy of dual B1R/B2R agonists-antagonists/TRIOZAN™ based NPs to enhance targeting of drugs such as mAbs to the brain.

EXAMPLE I

Peptide synthesis and purification

[0060] Peptides were assembled on 2-chlorotrityl-chloride resin (0.9 mmol/g by an automated Symphony-X peptide synthesizer (Protein Technologies, Inc.) using standard 9-fluorenylmethyloxycarbonyl (Fmoc) SPPS chemistry. Briefly, the couplings of 5 equivalents of Fmoc-protected amino acids were performed in the solution of

- 16 -

dichloromethane (DCM)/N, N-dimethylformamide (DMF) (1:1, v/v) using 5 equivalents of HATU and 9 equivalents of DIPEA for 1h. When applicable, the N-terminal Mpa residue was introduced as its Trt S-protected derivative. Fmoc groups were removed by treating the resin with 20% piperidine in DMF for 10 min (2x). The peptides were cleaved from resin by HFIP/ DCM (7/3) for 1h and deprotected using the cleavage cocktail of TFA: triisopropylsilane (TIPS): H₂O (95:2.5:2.5/ v:v:v) at room temperature for 3h. The solution of the released peptides was filtered, concentrated, and precipitated in cold diethyl ether. After centrifugation, the supernatants were removed and the thereafter, crude peptides were purified by analytical RP-HPLC (Waters 2535 module) on a C18 column (ACME C18, 10 µm, 250 x 30 mm, Canadian Life Science) using absorbance at 214 nm. Purified peptide fractions were pooled, lyophilized, and stored at -20°C. Stock solutions (10 mM) of peptides were also prepared in Nanopure water and then stored at -20°C until use. Purity and identity of purified peptides were evaluated using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-UV-MS, Waters AQUITY-H-Class-SQD2, column Waters BEH C18 (1.7 µm, 2.1 x 50 mm)). All synthesized peptides were > 98% pure, with expected mass spectra. Abbreviations for amino acids follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Other abbreviations are described as follows: Hyp, trans-4-hydroxy-L-proline; Thi, α-(2-thienyl)-L-alanine; Orn, L-ornithine; Oic, L-(2S,3aS,7aS)-octahydro-1H-indol-2-carboxylic acid; β-Nal, 3-(2-naphthyl)-alanine; Igl, 2-indanyl-glycine; NChg, N-cyclohexyl-glycine; Sar, N-methyl-glycine; (αMe)Phe, α-methyl-phenylalanine; Mpa, 3-mercaptopropanoyl.

Peptide conjugation on TRIOZAN™

[0061] 100 mg of Ts-TRIOZAN™ (TMC: N,N,N-trimethyl chitosan, Mw=65,000 Da, degree of quaternization: 60%, provided by Ovensa Innovations Inc.), 1 mL of TEA and 13 mg B1R agonist peptide were dissolved into 15 mL of water in a 100 mL round-bottom flask under inert condition (7 equivalent peptide/Trioan; TMC-B1R). The reaction mixture was kept under stirring at room temperature for 3 days. After the grafting reaction, the mixed solution was dialyzed against deionized water using a 12-14 kDa cut-off dialysis membranes (Spectrum Labs™) for 3 days at room temperature (with bath changes every day), then recovered by lyophilisation and stored at -20°C until further utilized.

Preparation of TRIOZAN™-based NPs for mAb loading

- 17 -

[0062] TRIOZAN™ chloride powder (TMC) was dissolved in autoclaved acetate buffer saline (pH 5.6, 10 mM, NaCl = 137 mM) to a final concentration of 3 mg/mL, and then filtered using a media filtration system (pore size: 0.45 µm). TRIOZAN™ solution was adjusted to pH > 9 using autoclaved bicarbonate buffer saline solution (pH 9.3, 0.1 M, NaCl 137 mM) by adding 20% volume. The mAb antibody stock solution (2.5 mg/mL in phosphate-buffered saline (PBS)) was diluted 1.2x with bicarbonate buffer saline (pH 9.3, 0.1 M, NaCl 137 mM) and incubated 10 min on ice. Thereafter, mAb was added dropwise into TRIOZAN™ to a final weight ratio of 1:30 antibody: TRIOZAN™. The peptide B1R agonist (NG29, final concentration 90 µM) and the B2R agonist (NG291, final concentration 45 µM) were subsequently added for the Formulation 6. For the preparation of the Formulation 4 comprising B1R agonist functionalized NPs, a TMC/TMC-B1R ratio of 9/1 was used instead of the free B1R peptide agonist. The TRIOZAN™ (+mAbs/B1R/B2R) solution and a solution of PBS (pH 7.4, 0.01 M, NaCl 137 mM) containing dextran sulfate (DS, Mw = 500,000 Da: 1 mg/mL): tripolyphosphate (TPP: 0.5 mg/mL), were loaded in two separate 10 mL syringes. Both syringes were placed in a NanoAssemblr® containing a sterile NanoAssemblr® NxGen microfluidic chip. The flow rate was set at 12 mL/min with a final wt/wt ratio between TRIOZAN™ and DS equal to 6:1. The freshly prepared NPs were centrifuged at 42,000 g for 45 min at 4°C. The pellets (clear with a yellowish tint) were resuspended in PBS using short bursts of sonication and filtered through 0.45 µm PVDF filters. Sucrose (final concentration: 50 mg/mL) was finally added to NP suspensions before freeze-drying.

Competition binding assays

[0063] Competition binding assays were carried out essentially as described previously (Sikpa et al., 2020, Pharmaceuticals (Basel), 13 (10)). Briefly, HEK293 cells stably expressing either human kinin B1R or B2R were grown in 24-well plates and incubated with 4 nM [³H]Lys[Leu⁸]desArg⁹BK (for B1R) or with 1 nM [³H]BK/well (for B2R) in serum-free DMEM for 1-2h in the absence or presence of increasing concentrations of competitors (10⁻¹¹-10⁻⁵ M for peptides; 10⁻¹⁰-10⁻⁴ g/mL for peptides-coupled NPs). Radioactivity of samples was measured by a β-scintillation counter (PerkinElmer). Binding affinities of compounds were expressed in terms of IC₅₀ values - the concentrations of competing compounds (i.e., free peptides, TMC-peptides conjugates and peptides coupled to NPs) causing 50% displacement of specific binding of the radioligand. Data are means of 2-3 independent experiments.

mAb protein determination

- 18 -

[0064] Absolute protein concentrations of mAbs alone or loaded in TRIOZAN™ based-NP formulations were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, Massachusetts, USA). Samples were measured for light absorbance at 280 nm and Beer's Law used to calculate concentration, with molar extinction coefficient (ϵ) equal to $210,000\text{M}^{-1}\text{cm}^{-1}$.

DLS analysis

[0065] The average sizes (nm) and polydispersity indices of TRIOZAN™ based-NPs were determined by dynamic light scattering (DLS) using the UNCLE-Unchained Labs system (Unchained Labs, Pleasanton, CA, USA). Ten μL of each antibody was loaded into the multi-micro cell array. The DLS measurement was recorded at 20°C . Ten readings were taken for each individual analysis, with outliers discarded, and the remaining data averaged. The Uncle software (version 2.0) correlation function was subsequently used to calculate DLS measurements (size distribution and polydispersity). Alternatively, DLS analyses of the mAbs loaded-TRIOZAN™ NPs were performed at room temperature using a Zetasizer Nano ZS (Malvern® Instrument).

Animals

[0066] Male and female double transgenic mice (B6C3-Tg (APP^{swe}/PSEN1^{dE9}); 9- to 12-month-old; bodyweight 35-50 g) were used in this study. Mice were purchased from the Jackson Laboratory (MMRRC Strain #034829-JAX). Animals were maintained under standard diurnal conditions and were allowed access to food and water ad libitum. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Université de Sherbrooke (protocol #2020-2508) and performed in accordance with the Canadian Council on Animal Care guidelines.

Biodistribution studies

[0067] Biodistribution studies of the naked mAb and the two mAb loaded-NP formulations were conducted following two routes of administration (IV, IN) in the transgenic mouse model of AD. Tissue samples (entorhinal cortex, hippocampus, and liver) and blood were collected 24h post-administration. The tested mAbs (undisclosed for confidentiality reason) was detected by targeted proteomics on its unique peptide sequence: SSGYHFTSYWMHWVK. The stable heavy isotope labeled counterpart (AQUA peptide: SSGYHFTSYWMHWV[K+8]) was used as an internal standard (ThermoScientific). A standard curve was established with a synthetic peptide and the

- 19 -

heavy labelled peptide in a peptide mixture at various concentrations. Absolute quantification experiments were conducted in tissue samples and the serum.

mAb administration and tissue collection

[0068] mAb formulations (dose: 10 mg/kg) were administered IV or IN in isoflurane anesthetized-mice (5% induction, 2% maintenance). For IV administration, formulations were given as a bolus (< 1 min) with a 50 µL saline flush via the caudal vein. For IN administered formulations, after rehydration of lyophilized powders, NP formulations were concentrated about 6- to 7-fold using 10 kDa MWCO ultrafiltration membranes (Amicon Ultra). A total volume of 5-6 µL was delivered into each nostril using a pipette over the course of 5 min. At the end of the experiments, blood and organ were collected from mice anesthetized with ketamine/xylazine (intramuscular injection, 87/13 419 mg/kg). Serum was prepared from blood harvested from cardiac puncture. Prior to organ harvesting, mice were euthanized by transection of the right atrium followed by an injection of 20 mL cold saline in the left ventricle to flush the blood from the brain and organs. Harvested tissue and serum samples were flash frozen in liquid nitrogen and stored at -80°C until analysis.

Mass spectrometry sample preparation

[0069] Tissue samples were prepared according to Delcourt et al. (2018, Mol Cell Proteomics, 17(12): 2402-2411) and Dubois et al. (2020, Nat Commun, 11(1): 1306). Briefly, tissue samples were homogenized using a Mini Bead Mill Homogenizer with 2.8 mm ceramic beads for hard tissue homogenization in 500 µL Lysis Buffer (8 M Urea, 10 mM HEPES pH 8.0). Lysates were sonicated to reduce viscosity followed by a 10 min centrifugation at 16,000 g to discard debris and insoluble parts. The supernatant was transferred in a LoBind tube and protein content was assessed using BCA protein assay. A total of 50 µg of protein in 50 µL of lysis buffer with 20 nM of the stable isotope peptide were reduced in 5 mM DTT, boiled 2 min at 95°C, rested 30 min at room temperature and alkylated in 7.5 mM 2-Chloroacetamide for 30 min in the dark at room temperature. The urea concentration in the lysate was reduced to 2 M with the addition of 50 mM NH_4HCO_3 and the samples were subjected to overnight trypsin digest (Trypsin Gold, MS Grade, Promega Corporation). Following digestion, the extracted peptides were acidified to a final concentration of 0.2% trifluoroacetic acid (TFA), desalted using C18 Zip-Tips, eluted with 200 µL of acetonitrile (ACN): H_2O : formic acid 440 (FA) (50:49:1), dried in a speedvac, and resuspended in 100 µL 1% FA in water.

- 20 -

LC-MS/MS analysis

[0070] A total of 10 μ L of peptide mixture was loaded and separated onto a nanoHPLC system (Dionex Ultimate 3000) with a constant flow of 4 μ L/min onto a trap column (Ac-claim PepMap100 C18 column, 0.3 mm id x 5 mm, Dionex Corporation). Peptides were then eluted off towards an analytical column heated to 40°C (PepMap C18 nano column (75 μ m x 25 cm)) with a linear gradient of 5–45% of solvent B (80% ACN with 0.1% FA) over a 42 min gradient at a constant flow (450 nL/min). Peptides were analyzed on an Orbitrap QExactive (Thermo Fischer Scientific) spectrometer using an EasySpray source at a voltage of 2.0 kV using PRM method. Acquisition of the MS/MS spectra was performed in the Orbitrap. An inclusion list containing the m/z values corresponding to the monoisotopic form of the normal and equivalent AQUA peptide (639.2910/641.9624 (3+) 453 and 479.7201/481.7236 (4+)) of the mAb was generated. The collision energy was set at 28% and resolution for the MS/MS was set at 35,000 for 200,000 ions with a maximum filling time of 110 ms with an insulation width of 2 and a chromatography peak width of 30s. Data acquisition was done using Xcalibur version 3.1.66.10.

Data and statistical analysis

[0071] Identification and quantification of the test mAb derived-peptide were performed on Skyline software (21.2.0.425). For quantification, the 6-7 most intense fragment ions were used for either the light peptide or the heavy peptide. Control experiments using similar brain tissues/serum samples from a mAb untreated B6C3-Tg AD mouse revealed minimal interference from background ions. These noise values were still subtracted from signal values in the MS data analysis.

Selected ions for liver quantification			
Heavy 3+	Light 3+	Heavy 4+	Light 4+
y4 577.3336 +	y9 619.2948 2+	y4 577.3336 +	y4 569.3194 +
y5 708.3741 +	y7 525.2549 2+	y3 440.2747 +	y6 296.1513 3+
y3 440.2747 +	y13 581.2696 3+	y5 708.3741 +	y9 310.1510 4+
y6 894.4534 +	b6 227.0993 3+	y6 894.4534 +	y13 871.4008 2+
b5 532.2150 +	b9 344.1470 3+	b6 340.1454 2+	y14 610.2803 3+
b6 679.2835 +	y13 871.4008 2+	b7 780.3311 +	y6 886.4392 +
b8 867.3632 +	y4 569.3194 +		
Selected ions for hippocampus and entorhinal cortex quantification			
Heavy 3+	Light 3+	Heavy 4+	Light 4+

- 21 -

y4 577.3336 1+	y9 619.2948 2+	y4 577.3336 +	y4 569.3194 +
b6 227.0993 3+	y7 525.2549 2+	y5 708.3741 +	y6 296.1513 3+
y5 708.3741 +	y13 581.2696 3+	y3 440.2747 +	y9 310.1510 4+
y3 440.2747 +	b6 227.0993 3+	y6 894.4534 +	y13 871.4008 2+
y6 894.4534 +	b9 344.1470 3+	b6 340.1454 2+	y14 610.2803 3+
b5 532.2150 +	y13 871.4008 2+	b7 780.3311 +	y6 886.4392 +
b6 679.2835 +	y4 569.3194 +		

Selected ions for serum quantification

Heavy 3+	Light 3+	Heavy 4+	Light 4+
y4 577.3336 +	y4 569.3194 +	y4 577.3336 +	y4 569.3194 +
b8 867.3632 +	b8 867.3632 +	y5 708.3741 +	y5 700.3599 +
y5 708.3741 +	y5 700.3599 +	y3 440.2747 +	y3 432.2605 +
y3 440.2747 +	y3 432.2605 +	y6 894.4534 +	y6 296.1513 3+
y6 894.4534 +	y6 886.4392 +	b6 340.1454 2+	b6 340.1454 2+
b5 532.2150 +	b5 532.2150 +	b7 780.3311 +	b7 780.3311 +
b6 679.2835 +	b6 679.2835 +		

[0072] The amount of the mAb protein was calculated using the light to heavy peptide ratio. Data are presented as mean $\pm$ SEM. Data were compared using two-way ANOVA with Sidak's correction. Statistical significance was set at $*p < 0.05$. Statistical analysis was performed using GraphPad 9.3.1 (GraphPad Software, Inc).

[0073] While the present description has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations and including such departures from the present disclosure as come within known or customary practice within the art and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

- 22 -

WHAT IS CLAIMED IS:

1. A nanoformulation comprising :
N,N,N-trimethyl chitosan (TMC);
a peptide agonist of kinin B1 receptor, a peptide antagonist of kinin B1 receptor
or a peptide agonist of kinin B2 receptor; and
a payload to be delivered across the blood-brain barrier (BBB).
2. The nanoformulation of claim 1, wherein the agonist of kinin B1 receptor, the
antagonist of kinin B1 receptor or the agonist of kinin B2 receptor is coupled to the
TMC.
3. The nanoformulation of claim 2, comprising a spacer coupling the agonist of kinin B1
receptor, the antagonist of kinin B1 receptor or the agonist of kinin B2 receptor to the
TMC.
4. The nanoformulation of claim 3, wherein the spacer is a Mpa, Mpa- β Ala, Mpa-Aca,
Mpa-PEG2, Mpa-PEG8, or Mpa-Gly.
5. The nanoformulation of any one of claims 1-4, comprising a ratio of 1: 7 of TMC:
B1R agonist.
6. The nanoformulation of any one of claims 1-5, comprising a B1R agonist coupled to
TMC and a non-cross-linked B2R agonist.
7. The nanoformulation of any one of claims 1-6, comprising a non-cross-linked B1R
agonist and a non-cross-linked B2R agonist.
8. The nanoformulation of any one of claims 1-6, comprising a B1R antagonist coupled
to TMC and a non-cross-linked B2R agonist.
9. The nanoformulation of any one of claims 1-6, comprising a non-cross-linked B1R
antagonist and a non-cross-linked B2R agonist.
10. The nanoformulation of any one of claims 1-9, comprising at least one B1R agonist
selected from the group consisting of:

- 23 -

β Ala-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

β Ala-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Gly-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Gly-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Sar-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Sar-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Aca-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Aca-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH; and

Lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH.

11. The nanoformulation of any one of claims 1-7, comprising at least one B2R agonist selected from the group consisting of:

Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH;

Lys-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

β Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH; and

Aca-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH.

12. The nanoformulation of any one of claims 1-7, comprising at least one B1R antagonist selected from the group consisting of:

AcOrn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

β Ala-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

AcLys-Lys-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH;

- 24 -

Aca-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH; and

Lys-Orn-Arg-Oic-Pro-Gly-(α Me)Phe-Ser-D β Nal-Ile-OH.

13. The nanoformulation of any one of claims 1-12, wherein the agonist or antagonist of kinin B1 receptor further comprises a chelating compound at the N-terminus.

14. The nanoformulation of claim 13, wherein the chelating compound is 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA); 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA); methylhydroxamates derived from triaza- and tetraaza macrocycles (NOTHA2 and DOTHA2); 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA), or a derivative thereof.

15. The nanoformulation of any one of claims 1-14, comprising at least one B1R agonist, B2R agonist, or B1R antagonist selected from the group consisting of:

Mpa- β Ala-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Mpa- β Ala-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Mpa-Gly-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Mpa-Gly-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

Mpa-Aca-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

Mpa-Aca-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-Pro-DPhe-OH;

NOTA-Aca-Lys(Mpa)-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-DPhe-OH;

H-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

H-Lys-Arg-Pro-Hyp-Gly-Phe-Cys-Pro-Phe-Arg-OH;

Mpa- β Ala-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

H-Lys(Mpa)-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

Mpa- β Ala-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH;

- 25 -

Mpa-Aca-Arg-Pro-Hyp-Gly-Thi-Ser-^NChg-Thi-Arg-OH;

Mpa-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH;

Mpa-PEG2-βAla-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH;

AcLys(Mpa)-Lys-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH;

Mpa-Aca-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH; and

NOTA-Aca-Lys(Mpa)-Orn-Arg-Oic-Pro-Gly-(αMe)Phe-Ser-DβNal-Ile-OH.

16. The nanoformulation of any one of claims 1-15, comprising a ratio of 2:1 of agonist or an antagonist B1R ligand: agonist or an antagonist of B2R.

17. The nanoformulation of any one of claims 1-16, wherein the agonist of kinin B1 receptor, the antagonist of kinin B1 receptor or the agonist of kinin B2 receptor further comprises a radiolabeling agent at its N-terminus.

18. The nanoformulation of claim 17, wherein the radiolabeling agent is ⁶⁴Cu, ⁶⁷Cu, ⁶⁸Ga, ¹³¹I, ¹¹¹In, ¹⁵³Sm, ⁸⁹Sr, ⁹⁰Y, ¹⁷⁷Lu, ²²⁵Ac or ²¹³Bi.

19. The nanoformulation of any one of claims 1-18, wherein the payload is siRNA, a shRNA, an antibody, a small molecule, an antisense, a polynucleotide, or an aptamer.

20. The nanoformulation of claim 19, wherein the antibody is a monoclonal antibody, a polyclonal antibody or a humanized antibody.

21. The nanoformulation of any one of claims 1-20, formulated for an intravenous (IV) administration, intranasal (IN) administration, intraarterial administration or convection-enhanced delivery (CED).

22. The nanoformulation of any one of claims 1-21, for the treatment of a central nervous system (CNS) disease.

23. The nanoformulation of any one of claims 1-22, for measuring the biological effects of the payload to treat a central nervous system (CNS) disorder.

- 26 -

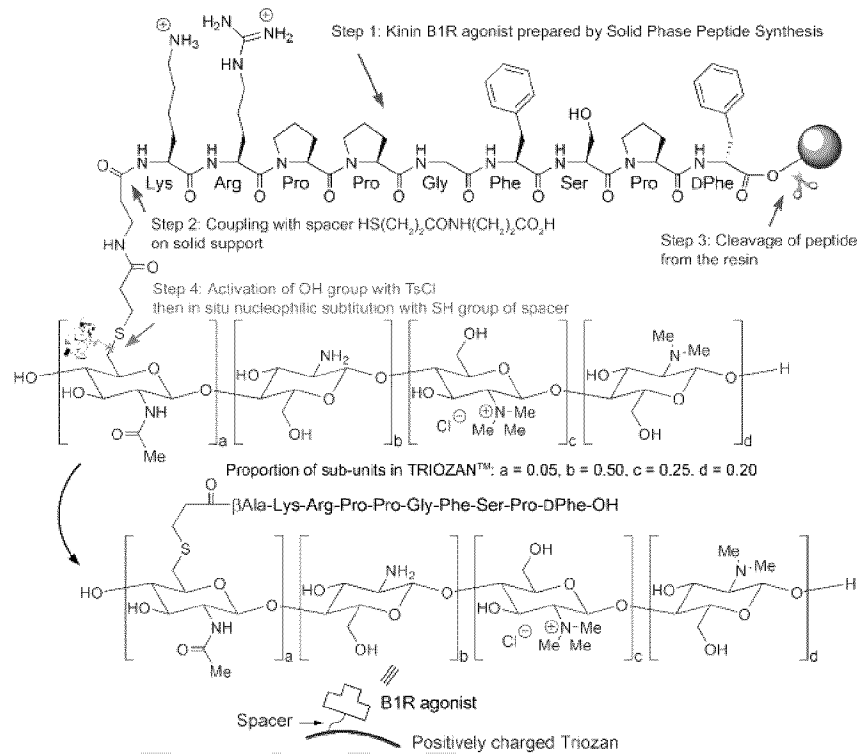
24. A method of treating a central nervous system (CNS) disorder comprising the step of administering the nanoformulation of any one of claims 1-23.

25. The method of claim 24, wherein the nanoformulation is administered intravenously or intranasally, or by a convection-enhanced delivery (CED).

26. Use of the nanoformulation of any one of claims 1-21 for treating a central nervous system (CNS) disorder.

27. Use of the nanoformulation of any one of claims 1-21 in the manufacture of a medication for treating a central nervous system (CNS) disorder.

1/3



1

Fig. 1

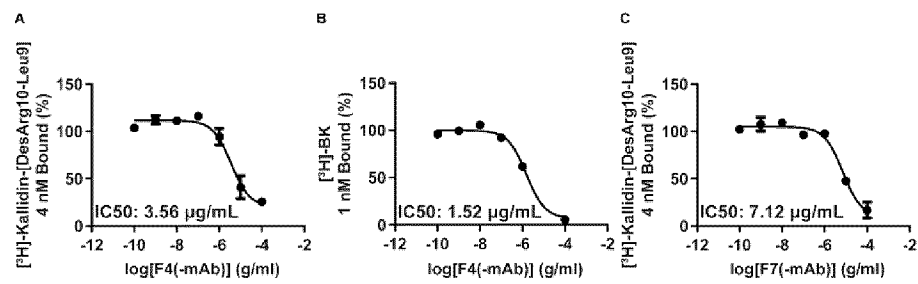


Fig. 2

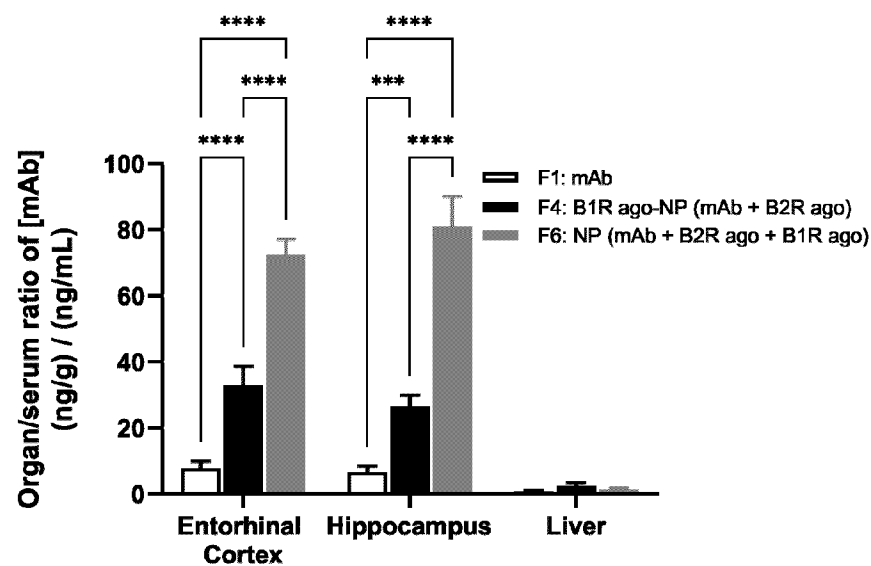


Fig. 3

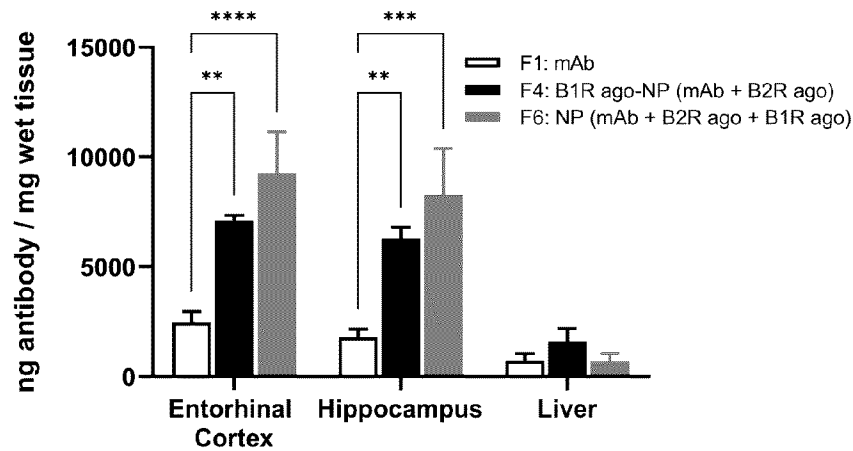


Fig. 4

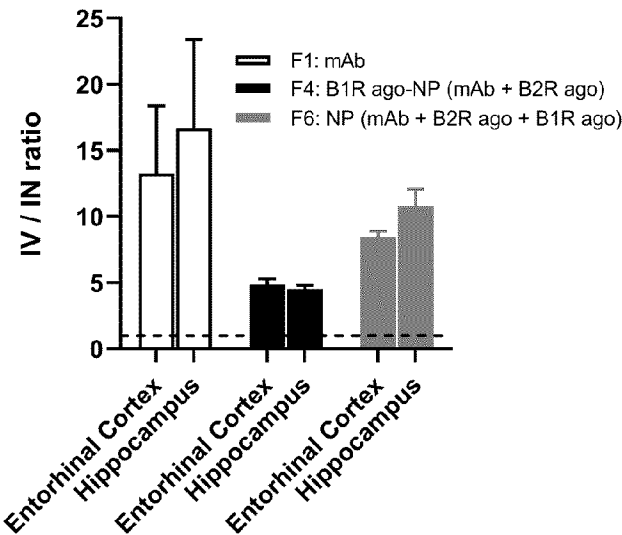


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2024/050823

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 47/69** (2017.01), **A61K 9/51** (2006.01), **A61K 39/395** (2006.01), **A61K 47/62** (2017.01), **A61P 25/00** (2006.01), **C07K 7/06** (2006.01), **C07K 9/00** (2006.01)

CPC: **A61K 47/6939** (2020.01), **A61K 39/395** (2020.01), **A61K 47/62** (2020.01), **A61K 47/6939** (2020.01), **A61K2121/00** (2020.01), **A61P 25/00** (2020.01), **C07K 7/06** (2020.01), **C07K 9/001** (2020.01)

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)

IPC: **A61K 47/69** (2017.01), **A61K 9/51** (2006.01), **A61K 39/395** (2006.01), **A61K 47/62** (2017.01), **A61P 25/00** (2006.01), **C07K 7/06** (2006.01), **C07K 9/00** (2006.01)

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

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

Questel Orbit, STNext, PubMed, Scopus, Google (Keywords including N,N,N-trimethyl chitosan, TRIOZAN™, kinin B1, kinin B2, peptide, agonist, antagonist, blood-brain barrier, central nervous system disorder and related terms)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GAGNON et al., "Evaluation of novel B1R/B2R agonists containing TRIOZAN™ nanoparticles for targeted brain delivery of antibodies in a mouse model of Alzheimer disease", <i>Molecule</i> , 4 July 2023, 28, 5206. (see entire document)	1-27
Y	DUARTE-GUTERMAN et al., "Oxytocin has sex-specific effects on social behavior and hypothalamic oxytocin immunoreactive cells but not hippocampal neurogenesis in adult rats" <i>Hormones and Behavior</i> , 2020, 122, 104734. (see section 3.4)	1-27
Y	KUMAR et al., "Evaluation of neuropeptide-loaded trimethyl chitosan nanoparticles for nose to brain delivery", <i>International Journal of Biological Macromolecules</i> , 2013, 61, 189-195. (see entire document)	1-27

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* "A" "D" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance document cited by the applicant in the international application earlier application or patent but published on or after the international filing date 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) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	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 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 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
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Date of the actual completion of the international search
06 September 2024 (06-09-2024)

Date of mailing of the international search report
09 September 2024 (09-09-2024)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 819-953-2476

Authorized officer

Wesley Sharman (819) 639-9360

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2024/050823

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GAO et al., "RVG-peptide-linked trimethylated chitosan for delivery of siRNA to the brain", <i>Biomacromolecules</i> , 2014, 15, 1010-1018. (see entire document)	1-27
Y	CAPRIFICO et al., "Overcoming the blood-brain barrier: Functionalised chitosan nanocarriers", <i>Pharmaceutics</i> , 2020, 12, 1013. (see section 4.1)	1-27
Y	WO2012171125 (GAGNON) 20 December 2012 (20-12-2012) (see entire document)	1-27
Y	WO2006128293 (GOBEIL et al.) 7 December 2006 (07-12-2006) (see entire document)	1-27
Y	CÔTÉ et al., "Dual kinin B1 and B2 receptor activation provides enhanced blood-brain barrier permeability and anticancer drug delivery into brain tumors", <i>Cancer Biology & Therapy</i> , 2013, 14(9), 806-811. (cited in description) (see entire document)	1-27
Y	CÔTÉ et al., "Induction of selective blood-tumor barrier permeability and macromolecular transport by a biostable kinin B1 receptor agonist in a glioma rat model", <i>PloS ONE</i> , 2012, 7(5), e37485. (see entire document)	1-27
Y	WO2016205941 (GOBEIL et al.) 29 December 2016 (29-12-2016) (see entire document)	1-27
Y	WO2006128293 (GOBEIL et al.) 7 December 2006 (07-12-2006) (see entire document)	1-27
A	WO9807746 (REGOLI et al.) 26 February 1998 (26-02-1998) (see entire document)	1-27
A	KULHARNI et al., "N,N,N-trimethyl chitosan : An advanced polymer with myriad of opportunities in nanomedicine", <i>Carbohydrate Polymers</i> , 2017, 157, 875-902.	1-27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2024/050823**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. ☒ forming part of the international application as filed.
- b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13^{ter}.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2024/050823

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2012171125A1	20 December 2012 (20-12-2012)	CA2869821A1	20 December 2012 (20-12-2012)
		CA2869821C	19 June 2018 (19-06-2018)
		EP2718331A1	16 April 2014 (16-04-2014)
		EP2718331A4	15 April 2015 (15-04-2015)
		EP2718331B1	02 August 2017 (02-08-2017)
		ES2644138T3	27 November 2017 (27-11-2017)
		JP2014518289A	28 July 2014 (28-07-2014)
		JP6346561B2	20 June 2018 (20-06-2018)
		JP2017222869A	21 December 2017 (21-12-2017)
		US2014107329A1	17 April 2014 (17-04-2014)
		US9505852B2	29 November 2016 (29-11-2016)
WO2006128293A1	07 December 2006 (07-12-2006)	EP1937710A1	02 July 2008 (02-07-2008)
		EP1937710A4	06 January 2010 (06-01-2010)
		EP1937710B1	17 October 2012 (17-10-2012)
		US2009054347A1	26 February 2009 (26-02-2009)
		US8076453B2	13 December 2011 (13-12-2011)
WO2012129774A1	24 May 2012 (24-05-2012)	US2012129774A1	24 May 2012 (24-05-2012)
WO2016205941A1	29 December 2016 (29-12-2016)	CA2990053A1	29 December 2016 (29-12-2016)
		EP3313861A1	02 May 2018 (02-05-2018)
		EP3313861A4	20 March 2019 (20-03-2019)
		JP2018524401A	30 August 2018 (30-08-2018)
		JP7091241B2	27 June 2022 (27-06-2022)
		US2018311385A1	01 November 2018 (01-11-2018)
		US11213597B2	04 January 2022 (04-01-2022)
WO2006128293A1	07 December 2006 (07-12-2006)	EP1937710A1	02 July 2008 (02-07-2008)
		EP1937710A4	06 January 2010 (06-01-2010)
		EP1937710B1	17 October 2012 (17-10-2012)
		US2009054347A1	26 February 2009 (26-02-2009)
		US8076453B2	13 December 2011 (13-12-2011)
		US2012129774A1	24 May 2012 (24-05-2012)
WO9807746A1	26 February 1998 (26-02-1998)	AU3935897A	06 March 1998 (06-03-1998)
		US7041785B1	09 May 2006 (09-05-2006)