Electronic Supplementary Information

Modifications in the Chemical Structure of Trojan carriers: Impact on Cargo Delivery

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A - Peptide and pseudo-peptide synthesis

Amounts of reagents are given in equivalents with respect to the free amino groups of the peptidyl-resin unless mentioned otherwise. All coupling, deprotection and functionalization protocols are given page S3.

Peptides:

Peptides (Arg_9, Lys_9, Pen, Kno, PKCi) were assembled by stepwise solid-phase synthesis on a ABI 433A peptide synthesizer (Applied Biosystems) using the standard Boc strategy (MBHA-PS resin, 0.9 mmol NH_2/g, amino acid activation with DCC/HOBt or HBTU) on 0.1 mmol scale. Myristoylation was achieved manually. Peptides were cleaved from the resin by treatment with HF. They were purified by reverse phase HPLC on a C8 column.

Pseudo-peptides:

Pseudo-peptide carriers were synthesized manually on a APOS 1200 parallel synthesizer (Rapp Polymere) using the Boc chemistry on MBHA-PS resin (0.05 mmol scale, resin loading: 0.25 mmol NH_2/g for compounds L2 to L5 and B1 and 0.12 mmol NH_2/g for D1 and D2). For all compounds, the synthesis started with the coupling onto the resin of Boc-Cys(4-MeOBzl)-OH and then Boc-

βAla-OH (or Boc-Gly-OH) using 10 equiv. of amino acid activated by treatment for 5 min with HBTU (9 equiv.) and DIEA (20 equiv.) in DMF (final concentration of activated amino acid: 0.45 M). Each coupling was allowed to proceed for 30 min under a stream of argon at room temperature. The sequence of residue assembly that was then followed for each compound is given below. The synthesis of L1-PKCi has already been described.[1]

- Compound L2 was synthesized by the consecutive assembly of four BocβAla-bisOrn(Alloc)$_2$-OH units. The *N*-terminal amino group was deprotected and acetylated. The δ amino groups of the "bis-ornithine" residues were then deprotected and functionalized by guanidinium. The peptide was cleaved from the resin by HF treatment.

- Compound L3 was synthesized by the consecutive assembly of three BocβAla-bisOrn(Alloc)$_2$-OH units and one BocGly-bisOrn(Boc)$_2$-OH. All amine protecting groups were removed (Alloc and Boc) and Boc-βAla-OH was coupled. After Boc removal, amines were functionalized by guanidinium. The peptide was then cleaved from the resin.

- Compounds L4 and L5 were synthesized by the coupling of BocβAla-bisOrn(Alloc)$_2$-OH unit(s) (four for L4, one for L5). A myristyl group was incorporated on the *N*-terminus after Boc removal. The side-chains of "bis-ornithine" were then deprotected and functionalized with guanidinium. The peptide was cleaved from the resin.

- Compound B1: After introduction of BocβAla-OH, a BocβAla-bisOrn(Alloc)$_2$-OH unit was coupled. The Boc group was removed and FmocβAla-OH was coupled. The peptidyl-resin was then treated with tetrakis(triphenylphosphine)palladium to remove the Alloc groups. It should be noted that during the washing steps that follow the Pd treatment, basic solutions should be avoided in order to keep the δ amino groups of the "bis-ornithine" protonated. Otherwise, those amino groups were found to remove the terminal Fmoc group. Both side-chains of "bis-ornithine" were then lengthened in parallel with BocβAla-OH, followed by Boc-Cys(4-MeOBzl)-OH coupling and acetylation. The Fmoc group was removed by treatment of the peptidyl-resin (3 x 3 min) with a solution of piperidine in DMF (20%, v/v). The pseudo-peptide was then elongated following the sequence described for compound L4.

- Compound D1: A first BocGly-bisOrn(Boc)$_2$-OH unit was introduced to form the core of the dendrimer. The three amino groups were deprotected and reacted with Boc-βAla-OH. Three BocGly-bisOrn(Boc)$_2$-OH units were then introduced in parallel, followed by amine deprotection and parallel coupling of (nine) Boc-βAla-OH. After Boc removal, guanidination was performed. The peptide was then cleaved from the resin.

- Compound D2: A BocGly-bisOrn(Boc)$_2$-OH unit was introduced to form the core of the dendrimer. Boc groups were removed, three Boc-βAla-OH were introduced in parallel. This was followed by the parallel coupling of three BocβAla-bisOrn(Alloc)$_2$-OH. After Boc removal, three myristyl groups were introduced simultaneously. The side-chains of "bis-ornithine" were deprotected and guanidination was performed. The peptide was cleaved from the resin.

Protocols:

- Coupling of BocβAla-bisOrn(Alloc)$_2$-OH or BocGly-bisOrn(Boc)$_2$-OH: dipeptide units (5 equiv.) were mixed with PyAOP (5 equiv.), HOAt (5 equiv.) and DIEA (20 equiv.) in DMF (final concentration of amino acid: 0.29 M) before addition to the peptidyl-resin. Each coupling was allowed to proceed for 12h at 50°C under a stream of argon. Double coupling were used. Completion of the reaction was checked by the Kaiser test. If necessary, capping of the unreacted amino groups was performed by treatment of the peptidyl-resin with a solution of acetic anhydride in DMF (10% v/v) for 30 min. The Boc protecting group was removed by treatment with trifluoroacetic acid for 3 min.

- Coupling of BocβAla-OH, FmocβAla-OH or BocGly-OH: amino acids (10 equiv.) were mixed with PyAOP (10 equiv.) and DIEA (20 equiv.) in DMF (final concentration: 0.5 M). Each coupling was allowed to proceed for 1 h under a stream of argon at room temperature.

- Myristoylation: myristic acid (20 equiv.) was mixed with PyAOP (20 equiv.), DIEA (25 equiv.) in DMF (final concentration: 0.85 M). Coupling was allowed to proceed under a stream of argon at room temperature overnight.

- Alloc removal: peptidyl-resin was treated with $Pd(PPh_3)_4$ (n equiv., n = number of Alloc groups) suspended in a mixture of DMF/$CHCl_3$/NMM/CH_3COOH (1.85/1.85/1/2, [Pd^0] = 50 mM) for 2 h in the dark under a stream of argon. The peptidyl-resin was then washed successively with DIEA in DMF (5%, v/v), DMF, diethyldithiocarbamate trihydrate in DMF (0.5% w/v), CH_2Cl_2 and DMF.

- Guanidination: 1H-Pyrazole-1-carboxamidine monohydrochloride (2.8 mmol) was mixed with DIEA (500 µL) in DMF (1.5 mL, final concentration 1.4 M) for 2h15 under a stream of argon at 50°C. The peptidyl-resin was washed with DMF. Complete guanidination reaction was characterized in the Kaiser test by reddish or red beads.

- **Acetylation**: the peptidyl-resin was treated with a solution of acetic anhydride in DMF (10%, v/v) for 30 min at room temperature under a stream of argon.

- HF cleavage: The peptide or pseudo-peptide was cleaved from the resin by treatment with anhydrous HF (1h30, 0°C) in the presence of anisole (1.5 mL/g peptidyl-resin) dimethylsulfide (0.25 mL/g peptidyl-resin) and, in the case of compounds containing 4-MeOBzlCys, *p*-toluenethiol (300 mg/g peptidyl-resin). After HF evaporation, the peptide was precipitated in cold ether and the resin was eliminated by filtration. The peptide was suspended in 10% degassed acetic acid and freeze-dried.

(See part B for pseudo-peptide purification)

B – Synthesis and characterization of carrier-cargo conjugates

Pseudo-peptide carriers L4, B1, D2 were purified by reverse-phase HPLC (RP-C8 column, using linear gradients of CH_3CN in 0.1% TFA) before conjugation *via* a disulfide bridge to the PKCi cargo containing a Cys(NpyS). Pseudo-peptides L2, L3, L5 and D1 were conjugated as crude mixtures to the PKCi cargo and then purified by reverse-phase HPLC. Noteworthy, in all cases, when the crude mixtures were analysed by HPLC, the expected compound was always found to correspond to the major peak.

- Protocol 1 of conjugation: crude carriers D1, L2, L3 and purified carriers L4 and $MyrArg_9$ (1µmol) were dissolved in a degassed solution of 10% acetic acid (100 µL) and mixed with the pure PKCi-Cys(Npys) compound (1.5 µmol, about 1.5 equiv.). The reaction was monitored by HPLC (reaction reaches completion in about 24h) and conjugates were purified by reverse-phase HPLC on a C8 column, using a linear gradient of acetonitrile in an aqueous solution containing 0.1% (v/v) TFA.

- Protocol 2 of conjugation,: crude carrier L5 and purified carriers B1, D2, MyrKno, MyrPen (1µmol) were dissolved in a degassed solution of DMSO (50 µL) and mixed with the cargo PKCi-Cys(Npys) dissolved in 50 µl of degassed 10% acetic acid (1.5 µmol, about 1.5 equiv. except for B1 which was mixed with 10 µmol, about 3 equiv./thiol group). The reaction was monitored by HPLC (reaction reaches completion in about 24h) and conjugates were purified, after dilution of the reaction milieu with a solution of 10% acetic acid (1 mL), by reverse-phase HPLC on a C8 column, using a linear gradient of acetonitrile in an aqueous solution containing 0.1% (v/v) TFA.

All conjugates were obtained with more than 95% purity as determined by HPLC analysis (isocratic conditions). They were characterized by MALDI-TOF MS in the positive ion reflector mode (except when otherwise mentioned) using CHCA matrix. Analyses were performed on a ABI 4700 Proteomics Analyser mass spectrometer (Applied Biosystems). The m/z values (first isotope) of the protonated CPP-PKCi conjugates are given below (the first line corresponds to the m/z of the free PKCi after removal of the NPys group).

Table S1. Characterization of the conjugates with CPPs

Peptides	**Cargo and CPP sequences**	**$[M+H]^+_{calcd}$**	**$[M+H]^+_{exp}$**
PKCi	BiotGGGGCRFARKGALRQKNV-NH_2	2100.1	2100.1
PKCi-Pen[(a)]	Ac-CRQIKIWFQNRRMKWKK-NH_2	4489.4	4490.0
PKCi-MyrPen	Myr-CRQIKIWFQNRRM$(O)_2$KWKK-NH_2	4687.7	4687.7
PKCi-Kno[(a)]	Ac-CKQINNWFINQRKRHWK-NH_2	4442.2	4442.1
PKCi-MyrKno	Myr-KQINNWFINQRKRHWKC-NH_2	4605.5	4605.6
PKCi-Arg_9[(a)]	Ac-CRRRRRRRRR-NH_2	3666.4	3667.1
PKCi-$MyrArg_9$	Myr-RRRRRRRRRC-NH_2	3833.2	3833.2

PKCi-Lys_9	Ac-CKKKKKKKKK-NH_2	3413.0	3412.9

Table S2. Characterization of conjugates with pseudo-peptides

Conjugates	t_R (min)[(b)]	$[M+H]^+_{calcd}$	$[M+H]^+_{exp}$
PKCi-L1	14.0	3228.8	3228.8
PKCi-L2	14.0	3636.0	3636.0
PKCi-L3	13.6	4261.4	4262.0
PKCi-L4	25.0	3804.2	3804.2
PKCi-L5	26.5	2825.6	2825.6
PKCi-B1[(a)]	22.0	8806.7	8805.8
PKCi-D1[(a)]	13.5	4435.2	4434.3
PKCi-D2	24.0	4339.7	4339.6

[(a)] MALDI-TOF MS Analysis was performed in the linear mode, the average m/z values are given in this case.

[(b)] HPLC retention times obtained with the linear gradient 0 to 100% B over A in 45 min (A: 0.1% TFA in H_2O; B: 0.1% TFA, 60% CH_3CN in H_2O), RP-C8 column.

C – Measure of the intact cargo delivered inside cells by MALDI-TOF MS

Principle:

Quantitation of the cargo delivered by the different carriers was performed by MALDI-TOF MS.[1,2] Peptide quantification is based on the use of an internal standard which corresponds to a peptide with the same sequence and labelled by deuterium. The cargo is functionalized by a biotin-Gly_4 isotope-labelling/affinity tag (non deuterated Gly for the internalized species (H-species), bi-deuterated Gly for the internal standard (D-species)). Cells are incubated with the different non-deuterated conjugates (H-PKCi-S-S-Carrier), a known amount of internal standard is added before cell lysis. Since the cargo is released when the internalized conjugate reaches a reducing compartment of the cell, we use as internal standard the free cargo (D-PKCI-SH). Recovery and purification of the biotinylated internalized cargo and standard is then performed using streptavidin-coated magnetic beads in reducing conditions (to reduce PKCi-S-S-Glutathione adducts formed during cell lysis[1]). Thus, for all conjugates it is always the free cargo that is detected and quantified by MALDI-TOF MS. For the reference corresponding to the cellular uptake of the free cargo (no carrier), the H-PKCi thioacetamide derivative was used to prevent dimerization during the internalization experiment (the deuterated thioacetamide PKCi was used as internal standard). This method gives the total amount of intact intracellular cargo. The cargo intracellular concentrations given in the manuscript were calculated with an estimated intracellular volume of 1.5 pL for a CHO cell.

Protocol:

The experiments were performed as reported previously.[2] Briefly, 10^6 adherent and confluent CHO-K1 cells were incubated with the conjugate (H-PKCi-S-S-Carrier, 7.5 μM in DMEM) for 75 min at 37°C. Cells were washed with DMEM, treated for 5 min at 37°C with 500 μL of a solution

containing 0.05% trypsin, 0.02% EDTA. Soybean trypsin inhibitor (100 µL, 5 mg/mL) and BSA (100 µL, 1 mg/mL) were added. The cell suspension was transferred in a microtube and the well was washed with 500 µL of 50 mM Tris–HCl buffer (pH 7.4). Both suspensions were pooled and centrifuged for 2 min at 640g. The pellet of intact cells was washed with 1 mL of 50 mM Tris-HCl buffer (pH 7.4), 0.1% BSA (buffer A) and centrifuged again. The pellet was mixed with a known amount of deuterated cargo (D-PKCi-SH) and 150 µL of 0.3% Triton X-100, 1 M NaCl and heated for 15 min at 100°C. The lysate was centrifuged for 5 min at 7,080 g. The supernatant was mixed with 850 µL of buffer A containing 2 mM DTT. The mixture was incubated for 1 h with 100 µg of streptavidin-coated magnetic beads (Dynabeads M-280, Dynal, France). Beads were washed,[2] mixed with 3 µL of a saturated solution of CHCA in acetonitrile/water-0.1% TFA (4:1) and left at RT for 10 min. After bead immobilization with a magnet, 1 µL of the elution mixture was deposited on the MALDI-TOF sample holder. The samples were analyzed by MALDI-TOF MS (ion positive reflector mode) on a 4700 Proteomics Analyser mass spectrometer or on a DE-Pro mass spectrometer (Applied Biosystems). The areas of the $[M+H]^+$ signals including all the isotopes of the intact H-PKCi and D-PKCi were measured (see insert Figure S3). The amount of internalized cargo was calculated from the area ratio. All internalization experiments were performed in duplicates and repeated three times independently.

Results:

The figure below summarizes the results obtained with the pseudo-peptide carriers (already presented in figure 2 of the manuscript) and the results obtained with the myristoylated CPPs MyrPen, MyrKno and $MyrArg_9$. The values obtained with Arg_9, Pen and Kno which have been previously published[1] have also been included to allow an easier comparison.

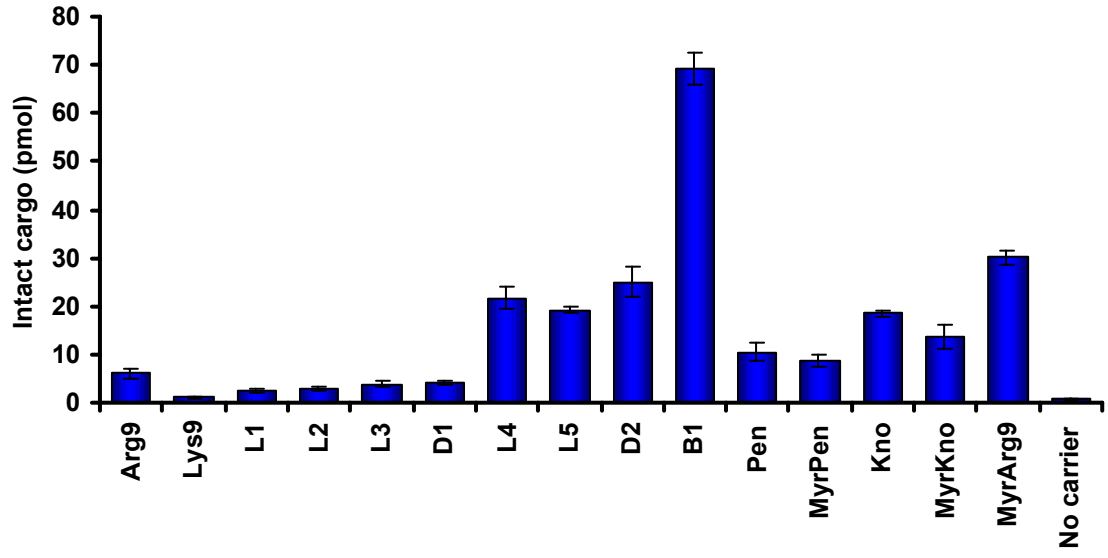


Fig. S1 Total amount of intact internalized PKCi cargo in 10^6 CHO cells. The carrier is indicated. Each data is the average result ± SEM from three independent experiments performed in duplicates. (Myr=myristoylated).

Incubation of the cells with the different conjugates in the indicated conditions did not provoke any cell death.

D - <u>Cytotoxicity assays</u>

The toxicity of the conjugates obtained with the new pseudo-peptide carriers was examined with a cell viability assay (CCK-8 cell counting kit):

Cell suspension (100 µL, 1500 CHO cells/well) were seeded in DMEM containing 10% FCS in 96-well microtiter plate at 37°C. Pseudopeptide-PKCi conjugates were added to the cells to give final concentrations of 1 or 7.5 µM (100 µL) and incubated for 75 min at 37°C. The CCK-8 cell counting kit was used according to the supplier's protocol (Dojindo Laboratories) and the absorbance was measured at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG LABTECH) with a reference wavelength at 620 nm. Experiments were performed in triplicates.

Conjugates were not found to be cytotoxic except for L5-PKCI which gave some toxicity at 7.5 µM (Figure S2). This was not observed in the experiments of MALDI-TOF MS quantification or confocal microscopy. The difference may be explained by the much higher peptide/cell ratio used in the cytotoxicity experiments (750 pmol peptide for 1500 CHO cells *i.e.* 500 fmol/cell) compared to the MALDI-TOF MS (7500 pmol peptide for 10^6 cells *i.e.* 7.5 fmol/cell) or CLSM experiments (500 pmol peptide for 10^5 cells *i.e.* 5 fmol/cell). The peptide/cell ratio is an important parameter since CPPs interact with cell membranes and in particular lipids of the membrane. This parameter is related to the peptide/lipid ratio which has been shown to be crucial in biophysical studies of CPPs.[3] The efficiency of CPP internalization has been shown previously to depend not only on the CPP concentration applied outside cells but also strongly on the CPP/cell ratio (keeping the CPP concentration constant).[4] Cytotoxicity is also likely to depend on the peptide/cell ratio for some carriers and their conjugates.

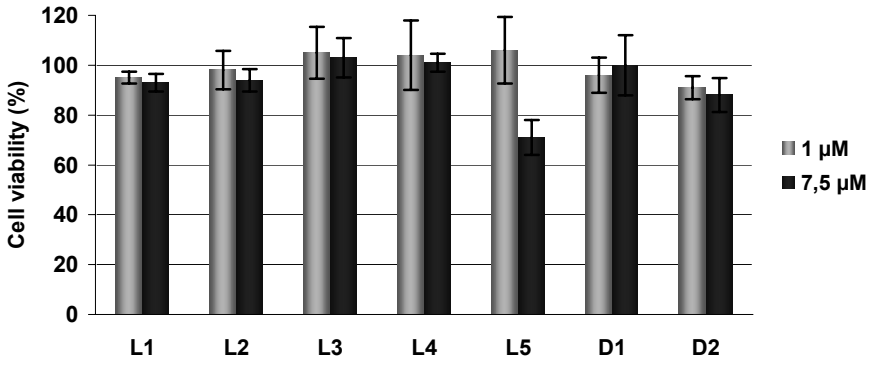


Fig. S2 Viability of cells incubated with the different conjugates PKCi-carrier at 1 or 7.5 µM. Values correspond to average ± SEM calculated from two independent experiments performed in triplicates.

E - Analysis of the proteolytic degradation of the PKCi cargo by MALDI-TOF MS

The method based on MALDI-TOF MS described part C also allows the detection and characterization of the biotinylated digests of the internalized cargo with m/z >600 (Figure S3).[1,2,5] It should be noted that for all the internalization experiments, no deuterated digest (from the internal standard D-PKCi) was detected showing that no artefactual degradation occurred during the process of peptide recovery and sample preparation.

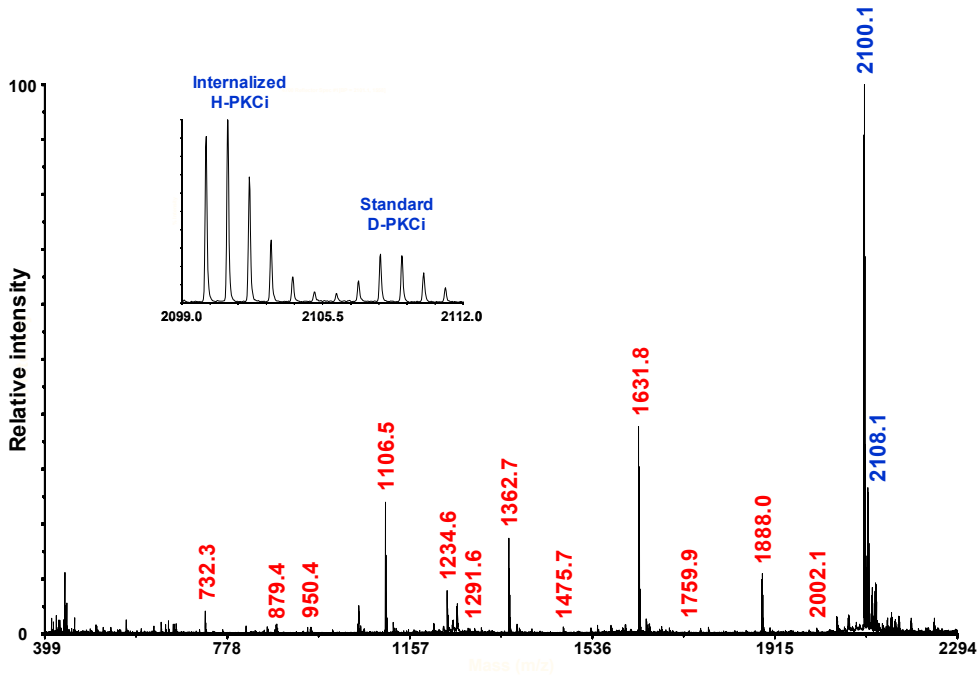


Fig. S3 MALDI-TOF mass spectrum obtained for the cellular uptake of the B1-PKCi conjugate showing the signals of the intact internalized cargo (H-PKCi) and internal standard (D-PKCi) (insert and m/z values written in blue) and the H-PKCi digests (red values). Mass spectrum was recorded on a 4700 Proteomics Analyser mass spectrometer (Applied Biosystems).

The absolute quantification of each digest of the internalized cargo would require the use of each corresponding internal standard (each digest labelled by a stable isotope added to the sample). However, since it is here always the same species, *i.e.* the free PKCi cargo, that is examined by MALDI-TOF MS, its profile of intracellular degradation can be compared when it is delivered by the different carriers. The relative ion abundance of each biotinylated peptide (intact H-PKCi and H-PKCi digests) was calculated as the ratio of its signal area to the total area of all signals (digests plus intact H-PKCi) (Figure S4).

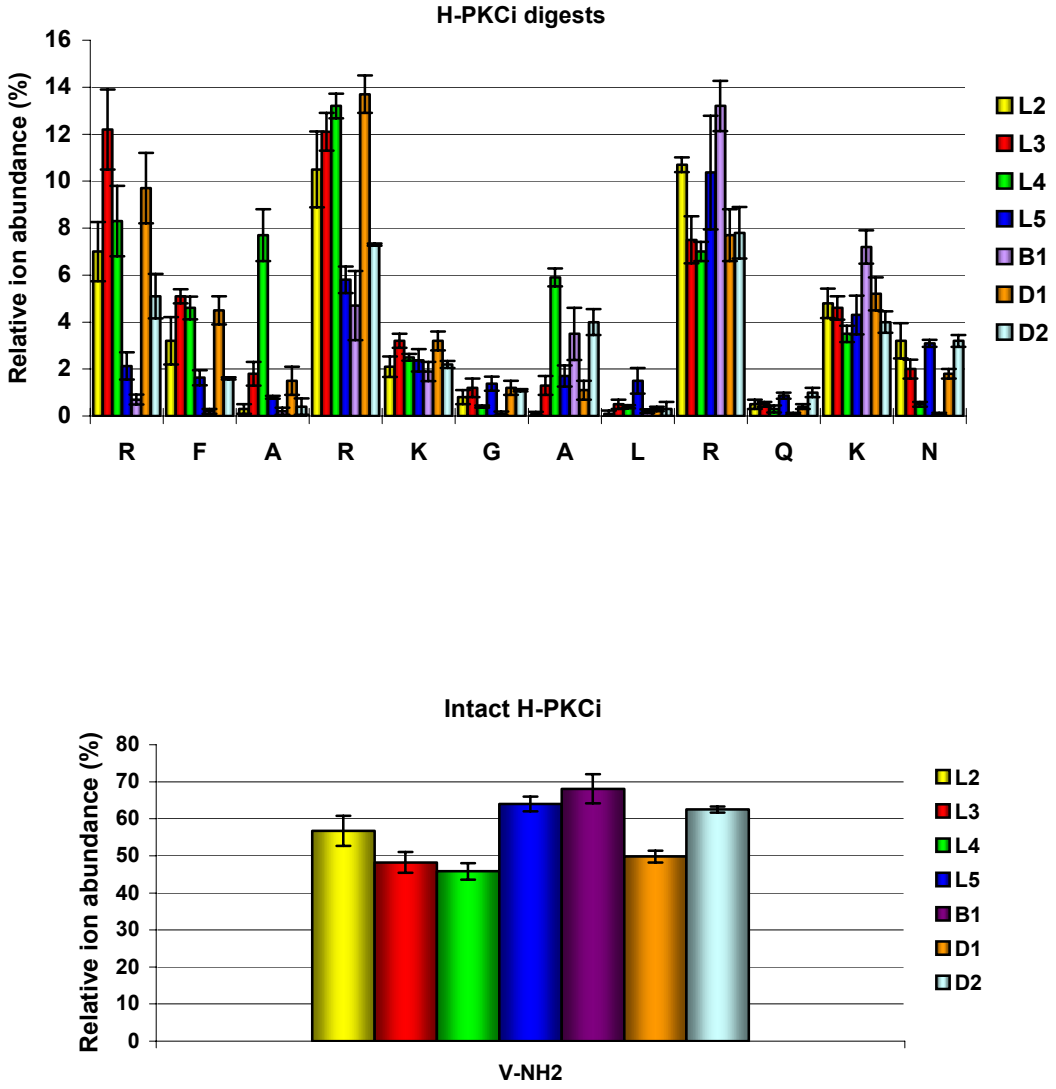


Fig. S4 Relative abundance of ions corresponding to the intact H-PKCi cargo and its digests depending on the pseudo-peptide carrier used for delivery, calculated from the MALDI-TOF mass spectra. Average values ± SEM calculated from three independent experiments. The *C*-terminal residue of each digest is indicated.

F - Analysis of the intracellular cargo localization by confocal laser scanning microscopy

Protocol:

CLSM was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss). CHO-K1 cells were plated on glass coverslips (10^5 cells, 1 cm^2) and cultivated for 24 hours. The cells were washed once with serum-free medium. Biotin-labelled conjugates (10 µM) were incubated with cells in 50 µL fresh serum-free DMEM/F12 medium for 30 min at the indicated temperatures (37°C and 4°C). Cells were washed three times with cold medium and incubated with unlabeled avidin (10 µM, 50µL) for 10 min at 4°C before fixation in 4% paraformaldehyde (20°C, 10 min) and permeabilization with 0.1% Triton X-100 in PBS (20°C, 5 min). Fixed cells were then incubated in

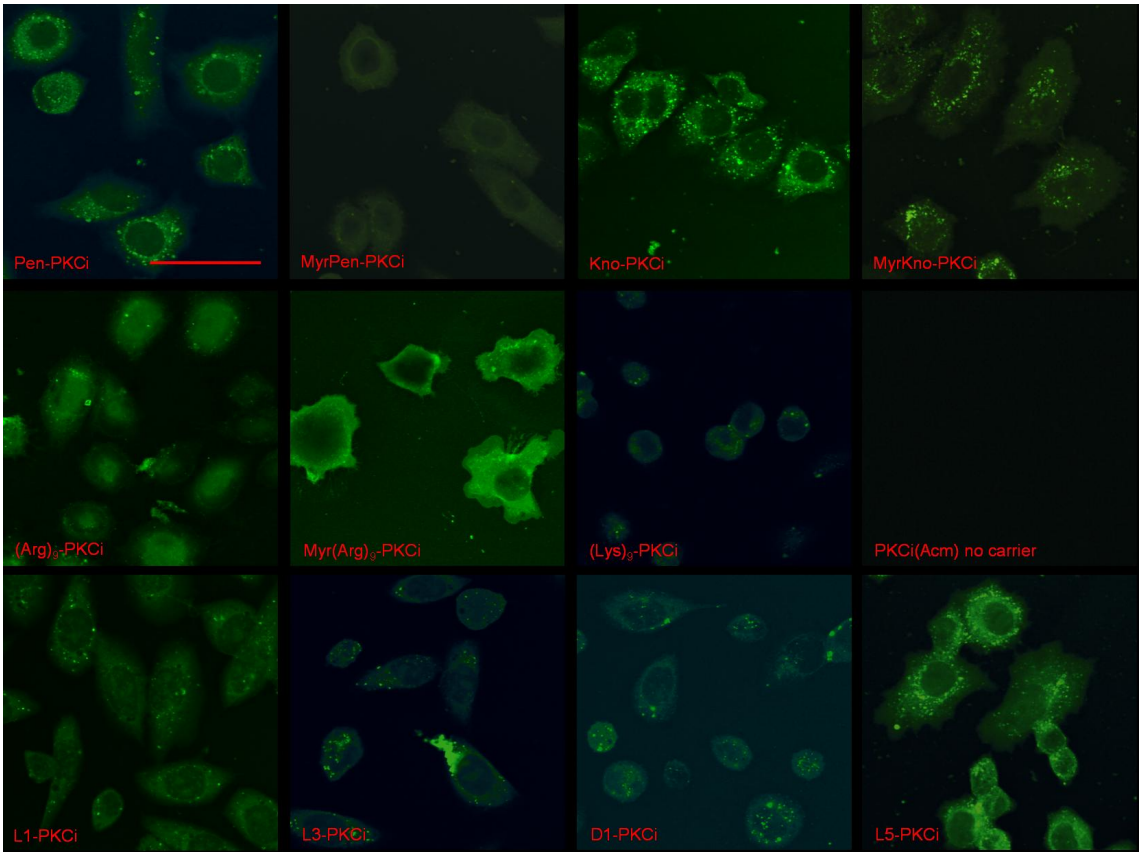
PBS with 10% foetal calf serum (20°C, 2h). Biotinylated peptides were detected with Alexa-488-Streptavidin (1µg/ml, 100 µL) in PBS (20°C, 30 min) and mounted in DAKO fluorescent mounting medium (DAKO Cytomation). Experiments were repeated at least 3 times independently with all conjugates tested in parallel.

Results:

The patterns of cargo distribution inside cells (Figures 4 and S5) were found to be highly reproducible. No cell death was observed when cells were incubated with the different conjugates in the conditions used for the CLSM experiments except with conjugates MyrKno-PKCi and MyrPen-PKCi which were found to be cytotoxic. These conjugates were thus studied by CLSM together with their parent non-myristoylated conjugates at 1 µM. All 4 carriers gave similar cargo localization in these conditions.

Cellular uptake of the conjugates was not totally inhibited at 4°C. Indeed, when incubated with cells at 4°C, all conjugates (except D2-PKCi) gave some diffuse cytosolic fluorescence. For the 4°C incubation, only the images obtained with the conjugate L4-PKCi and the free cargo *i.e.* no carrier are shown. The data obtained from conjugate incubation with cells at 37°C (labelled endosomes) and 4°C (diffuse cytosolic fluorescence) suggest that the conjugates are internalized both by temperature-dependent (endocytosis) and temperature-independent mechanisms (direct translocation of the plasma membrane).

Internalization at 37°C:



Incubation at 4°C:

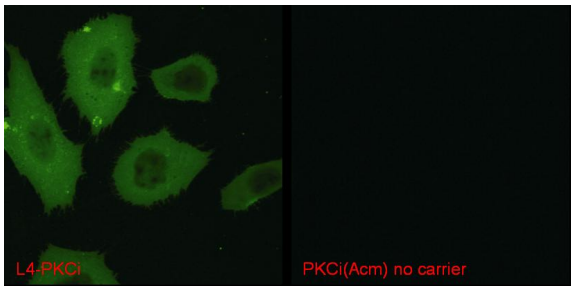


Fig. S5 Confocal microscopy images showing the cargo intracellular localization when delivered by the different carriers. Conjugate concentration = 10 μM except for MyrPen-PKCi and MyrKno-PKCi (1 μM). The scale bar represents 40μm.

Abbreviations:

Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; calcd, calculated; CHCA, α-cyano-4-hydroxycinnamic acid; CHO, Chinese hamster ovary; CLSM confocal laser scanning microscopy; CPP, cell-penetrating peptide; DCC, dicyclohexylcarbodiimide; DMEM, Dubelcco's modified eagle medium; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DTT, dithiotreitol; exp, experimental; FCS, foetal calf serum; EDTA, ethylenediamine tetraacetic acid; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 7-aza-1-hydroxybenzotriazole; HOBt, 1-hydroxybenzotriazole; H-PKCi, non deuterated PKCi; D-PKCi, deuterated PKCi; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBHA-PS, 4-methylbenzhydrylamine polystyrene; 4-MeOBzl, *p*-methoxybenzyl; Npys, 3-nitro-2-pyridinesulphenyl; PBS, phosphate-buffered saline; PyAOP, 7-Azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; PKCi, protein kinase C inhibitor; PKCi(Acm) thioacetamide derivative of PKCi; SEM, standard error of the mean; TFA, trifluoroacetic acid.

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