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RESEARCH ARTICLE

N-terminal modification of an LAH4-derived peptide increases mRNA delivery in the presence of serum

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The recently developed mRNA-based coronavirus SARS-CoV-2 vaccines highlighted the great therapeutic potential of the mRNA technology. Although the lipid nanoparticles used for the delivery of the mRNA are very efficient, they showed, in some cases, the induction of side effects as well as the production of antibodies directed against particle components. Thus, the development of alternative delivery systems is of great interest in the pursuit of more effective mRNA treatments. In the present work, we evaluated the mRNA transfection capacities of a series of cationic histidine-rich amphipathic peptides derived from LAH4. We found that while the LAH4-A1 peptide was an efficient carrier for mRNA, its activity was highly serum sensitive. Interestingly, modification of this cell penetrating peptide at the N-terminus with two tyrosines or with salicylic acid allowed to confer serum resistance to the carrier.

KEYWORDS

amphipathic peptide, cell penetrating peptide, gene delivery, LAH4 peptide family, mRNA transfection

1 | INTRODUCTION

The recently developed coronavirus SARS-CoV-2 vaccines highlighted the great therapeutic potential of mRNA in vaccination [1]. This first success has led to renewed interest in mRNA vaccination, which is now attracting massive industrial investment. In addition to the mRNA vaccines against SARS-Cov2, others are being evaluated in clinical trials: against cancer [2] and against rabies [3], and several other vaccines are in preclinical development such as vaccines against

pathogens like Zika virus [4, 5] and influenza [6]. The reasons for this attractiveness are multiple; not only is mRNA an excellent system for inducing the synthesis of any protein or peptide antigen, but it also has many advantages over plasmid DNA.

Major advances in mRNA vaccination efficiency have been made possible not only by modification of mRNA to increase its stability [7] but also by the development of efficient vectors to carry mRNA inside the cells. Indeed, mRNA is a polyanionic molecule that does not cross cellular membrane barriers. Therefore, the key to enabling the growth of this approach is the development of efficient vectors that complex, deliver and release mRNA into the cytosol. Most mRNA transfection systems are based on the use of lipid nanoparticles (LNPs) [8, 9], although some recent publications also describe the use of cell-penetrating peptides (CPPs) as delivery vehicle for mRNA [10–13].

Abbreviations: CMV, cytomegalovirus; CPP, cell-penetrating peptide; DLS, dynamic light scattering; FCS, fetal calf serum; LNPs, lipid nanoparticles; MTS, 3-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate buffer saline; PEI, polyethylenimine; RLU, relative light units; RPMI 1640, Roswell Park Memorial Institute.

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To note, the development of alternatives to LNPs is important because the use of LNP-based mRNA SARS-CoV-2 vaccines has led in some cases to the induction of side effects as well as the production of antibodies directed against particle components. Thus, developing next-generation mRNA-based nanoparticles for vaccination is a major concern. Peptides represent an interesting alternative for mRNA delivery for several reasons: Firstly, important parameters such as product identification and quality control are possible. Secondly, peptides offer a reproducible and scalable production process, and there is the possibility to include non-natural amino acids. Lastly, depending on the sequence and the amino acids that are used, it is possible to confer very different properties to a CPP: endosomolytic properties with the inclusion of histidines or other pH-sensitive groups such as 2,3-diaminopropionic acid [14], nuclear localization by adding NLS sequences [15], mitochondria [16] or surface receptor targeting [17], etc.

Our group has developed different types of cell penetrating peptides [18–20] and foldamers [21, 22] able to deliver efficiently DNA into cells. Among those CPPs, the cationic amphipathic histidine-rich LAH4 family stood out for its ability to transport a variety of cargoes including DNA [23–25], siRNA [26], proteins [27], peptide antigens [28], retroviruses [29–31], adeno-associated viruses [32], and quantum dots [33]. Now, in addition to those delivery capabilities, two recent reports showed that the LAH4-L1-derived LAH4 peptide also possesses mRNA delivery capacities [34, 35]. Moreover, in 2023, Xu et al. reported that a PEGylated LAH4-L1 peptide (PEG₁₂-LAH4-L1) efficiently delivers mRNA into tumor cells [36].

Based on these very promising results with LAH4-L1 and its PEGylated derivative, we wanted to evaluate whether other isomers of this CPP could be even more efficient for mRNA delivery. To address this question, we analyzed different peptides of the LAH4 family: The amino acid sequence was systematically changed to vary the number of leucine (LAH4-L) or alanine (LAH4-A) residues, respectively, and thereby also the size of the hydrophilic angle (Figure S1 and Table 1). LAH4-A1 derivative was the most efficient LAH4 derivative for mRNA transfection, but it is sensitive to serum present in media during the transfection step. To optimize transfection efficacy, this derivative was modified at the N-terminus. We found that tyrosine or salicylic acid modifications increased the mRNA transfection efficiency when serum was present.

2 | MATERIALS AND METHODS

2.1 | Peptides

Most of the LAH4 peptides (see Table 1) were purchased from Peptidicals. To exchange the counterions the peptides were solubilized three times in 2 mM hydrochloric acid and lyophilized. Some of the peptides were prepared in-house as previously described [37, 38]. The LAH4-A1 modified with either Trp2, Tyr2, or salicylic acid were synthesized by Proteogenix. All the peptides were dissolved in sterile Milli-Q water at 1 mg/ml and stored at –20°C.

TABLE 1 Sequence of the different peptides. The corresponding hydrophilic angle when presented in a Schiffer-Edmundson helical wheel projection is also shown (see Figure S1). The lysine residues are in bold black; the histidine residues (in cyan) delineate the hydrophilic angle (angle subtended by the positively charged histidine residues at pH 5); the amino acids present between the histidines within the hydrophilic face are either leucines (green) or alanines (magenta).

Name	Sequences	Hydrophilic angle
LAH4	KKALLALALHHLAHLALHLALALKKA-CONH₂	100°
LAH4-L0	KKALLAHALAHLALLALHLALHLKKA-CONH₂	60°
LAH4-L1	KKALLAHALHLLALLALHLAHLKKA-CONH₂	80°
LAH4-L2	KKALLALALHHLALLALHLAHLKKA-CONH₂	100°
LAH4-L3	KKALLALALHHLALLAHLAHLALALKKA-CONH₂	120°
LAH4-L4	KKALLHLALHAAALLAHLAHLKKA-CONH₂	140°
LAH4-L5	KKALLHLALHAAALLAHLAHLKKA-CONH₂	160°
LAH4-L6	KKALLHLALLAALHAHLAHLKKA-CONH₂	180°
LAH4-A1	KKALLAHALHLLAHLAHLAHLKKA-CONH₂	80°
LAH4-A2	KKALLAALHHLAHLAHLAHLKKA-CONH₂	100°
LAH4-A3	KKALLAALHHLAHLAHLAHLKKA-CONH₂	120°
LAH4-A4	KKALLAALHLLAHLAHLAHLKKA-CONH₂	140°
LAH4-A5	KKALLAALHLLAHLAHLAHLKKA-CONH₂	160°
LAH4-A6	KKALLAALHLLAHLAHLAHLKKA-CONH₂	180°

2.2 | Plasmid DNA and mRNA

The plasmid pSMD2LucΔITR 7.6 kbp was aliquoted at a concentration of 2 mg/ml in sterile Milli-Q water and stored at –20°C. This plasmid encodes for the *luciferase* gene pGL2 under the control of the human cytomegalovirus (CMV) immediate-early promoter. The following mRNA from TriLink Biotechnology (Tebu-Bio) were used: CleanCap® FLuc mRNA (Reference L-7602); CleanCap® EGFP mRNA (Reference L-7601) and CleanCap® Cyanine 5 Fluc mRNA (Reference L-7702).

2.3 | Cell culture

The four following human cell lines were used: HEK293T cells, which are human embryonic kidney cells expressing the large T antigen of simian virus 40; the human colorectal carcinoma cell line HCT116; the

human glioblastoma cell line U87; and the human immortalized keratinocytes HaCaT. The cell lines were cultured at 37°C in a humid atmosphere (5% CO₂) in RPMI 1640 (Roswell Park Memorial Institute) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (FCS).

2.4 | Preparation of the complexes

The peptide/DNA and peptide/mRNA complexes were prepared by mixing equal volumes of DNA (mRNA) and peptide in 150 mM NaCl or in a 10 mM phosphate buffer pH 6.25.

Alternatively, Hepes-buffered glucose (HBG) (20 mM Hepes pH 7.5, 5% glucose) or PBS (137.9 mM sodium chloride, 1.47 mM potassium phosphate monobasic, 2.67 mM potassium chloride, 8.09 mM sodium phosphate dibasic) was used. For example, 1.5 µg of DNA in 25 µl of 150 mM NaCl was mixed with increasing amounts of peptide in 25 µl of 150 mM NaCl. After vortex mixing for 20 s, the mixture was kept for 20 min at room temperature to allow the formation of the complexes. Culture medium was then added to obtain a final volume of 500 µl; next, 250 µl was added to each well of the duplicate. The peptide/mRNA complexes were prepared by following the same protocol as the one with DNA, with the exception that the amounts of nucleic acid and the volumes of preparation of the complexes were smaller; for example, for a duplicate, 500 ng of mRNA/15 µl of 150 mM NaCl was mixed with increasing amounts of peptide/15 µl of 150 mM NaCl.

2.5 | Size analysis by dynamic light scattering (DLS)

10 µg LAH4-A1-Tyr2 and 0.8 µg mRNA luciferase (or 12 µg LAH4-A1-Tyr2 and 1.5 µg DNA Luciferase) were each diluted in 25 µl of 150 mM NaCl. After the solutions were mixed and left for 20 min at room temperature, 50 µl of NaCl was added, and particle size was measured using a Zetasizer nano ZS (Malvern). The same protocol was used for measuring the size of the complexes prepared in phosphate buffer. The results are the average of three consecutive measurements. Particle size is expressed in intensity. Samples are considered as monodisperse if the polydispersity index (PDI) is <0.3.

2.6 | Transfection of plasmid DNA

The DNA transfection assays were done using 48-well plates. These experiments were done in biological duplicates (the same test was performed on two biological samples; i.e., we used two distinct wells/condition in the cell culture plates). Key experiments were done at least two times. The cells were seeded into the plates 1 or 2 days before transfection, in order to have a confluency of 60%–80%. For transfection, a serum-free culture medium or a medium with 10% serum was mixed with the complexes in order to have a final volume

of 250 µl per well. After removing the culture medium, the complexes were deposited into the wells, and the plates were placed into the incubator. After a period of 2.5 h of incubation, the transfection medium was replaced by fresh medium containing 10% serum. Then, the cells were incubated at 37°C until the end of the experiment (approx. 24 h). The luciferase and protein quantifications were done as described below.

2.7 | mRNA transfection

The mRNA transfection experiments were performed in 96-well plates. The HCT116, U87, and HEK293T cells were seeded into the plates the day before transfection in order to have a confluency of 60%–80%. The peptide/mRNA complexes were prepared as follows: mRNA in 15 µl of 150 mM NaCl (or phosphate buffer pH 6.25) were mixed with increasing amounts of carrier diluted in 15 µl 150 mM NaCl (phosphate buffer pH 6.25). The mixture was then kept for 15–20 min at room temperature to allow the formation of the complexes. Then 120 µl of serum-free culture medium (or medium with 10% serum) were added to the complexes in order to have a final volume of 150 µl. After removing the culture medium, the complexes were added to the cells (75 µl per well), and the plates were incubated at 37°C. After 2.5 h, the transfection medium was replaced by medium supplemented with 10% serum. Then, the cells were incubated at 37°C till the end of the experiment (around 24 h). The luciferase and protein quantifications were done as described below.

2.8 | Luciferase activity

The dosage of the luciferase was done as described [39] with slight modifications. Briefly, lysis buffer (8 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 0.6% Triton X-100, 15% glycerol, and 25 mM Tris-phosphate buffer pH 7.8) was added in each well after having first removed the culture medium. The lysates were then transferred into Eppendorf tubes and centrifuged for 5 min at 18,000 g. The dosage of the luciferase activity was realized with a luminometer (Berthold) after sequential addition of assay buffer (= lysis buffer without Triton but with ATP) and luciferin solution. The results are expressed in relative light units (RLU) and are normalized for the amount of proteins present in each sample and are expressed in RLU/1 s/mg protein. Of note, the light units/s measured with untreated cells are considered as background, and therefore, these values are subtracted from each sample. The amounts of protein were determined by a Bradford assay (Bio-Rad protein assay kit). This measurement was made using 4 µl of supernatant. To each sample was then added 200 µl of Bradford reagent diluted at 1/5 in Milli-Q water. The colorimetric assay was done by measuring the optical density (OD) at 595 nm using a spectrophotometer (SP2000, Safas). The amount of protein was determined using a calibration curve obtained using albumin as model protein.

2.9 | Cell viability

Cell metabolic activity was measured by using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay (CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega). MTS is a colorimetric reagent that undergoes color change in the presence of NAD(P)H, which is proportional to metabolic activity. The MTS assay was performed in duplicates using the following experimental conditions: 9500 HCT116 cells/well were seeded in 96-well plates in RPMI supplemented with 10% of serum. Two days later, the cells were transfected either in the absence or in the presence of 10% serum using different CPP/mRNA formulations. Twenty-four hours after transfection, 20 μ l per well of MTS reagent was added, followed by incubation at 37°C for 40 min (until significant color change was observed). Plates were read at 490 nm (reagent absorbance) and 680 nm (background) (SP200, Safas). The results were expressed by the subtraction of background absorbance and the expression of viability relative to non-treated controls (considered as 100% viability).

Alternatively, we also used a CellTiter-Glo 2.0 Cell viability Assay (#9241, Promega). This kit is based on cell metabolic activity by quantification of ATP released after cell lysis in the culture medium, which is directly proportional to the measured luminescence. In each well, the CellTiter-Glo reagent was added at a 1:1 ratio (v/v) to the medium and incubated for 3–5 min under agitation. Each supernatant was then transferred into a white 96-well plate, and another incubation of 15 min was done. To measure the luminescence, a microplate reader (Safas Monaco SP2000, Genius 5801) was used. Percentage of cell viability was defined by a percentage of the maximum viability (from the non-treated condition), and background luminescence was removed from the values from medium control alone.

2.10 | Flow cytometry and fluorescence microscopy

HEK293T cells plated in 48-well plates were transfected with LAH4-A1-Tyr2 peptides complexed to 1 μ g of mRNA encoding for GFP. After a 2.5-h incubation at 37°C in RPMI containing or not 10% serum, the medium was removed and replaced with fresh one. The GFP expression was evaluated 1 day post-transfection first by fluorescence microscopy and then by flow cytometry. For the cytometry analysis, cells were first washed once using PBS before addition of 100 μ l of a 1:1 mixture (v/v) of PBS/trypsin. Cells were then harvested, transferred in Eppendorf tubes, and centrifuged for 5 min at 350 g. The supernatants were removed, and the cell pellets were resuspended in 300 μ l of cold PBS. The Eppendorf tubes were kept on ice during the whole flow cytometry process. Flow cytometry data were analyzed with FlowJo software. For the experiments with the Cy5-labeled mRNA, the protocol was similar to that with the GFP mRNA except that flow cytometry was done after the 2.5 h of incubation with the complexes.

3 | RESULTS

3.1 | Design and evaluation of the LAH4-An and LAH4-Ln series

Two series of peptides which have been previously designed were studied [40]. The peptides, named LAH4-Ln and LAH4-An, are all isomers of LAH4. They are composed of the same 26 amino acids than LAH4: two lysines at both termini and a central core of eight alanines, 10 leucines, and four histidines to form the amphipathic helix. They however differ by the number (n) of leucines or alanines that are between the histidines when represented as Schiffer–Edmundson helical wheel (Figure S1). The amino acid modifications from the original LAH4 sequence [41] result in hydrophilic angles ranging from 60° to 180° (Table 1 and Figure S1).

The LAH4-An and LAH4-Ln peptides have been previously evaluated on human glioblastoma U87 cells for their DNA transfection capabilities [40]. Figure S2 shows the best transfection condition for each peptide (using a 10 mM acetate buffer pH 5 to generate the complexes). The best activity is observed for LAH4-L1 and LAH4-A1, which both have a hydrophilic angle of 80°. Using the same cell line and the same experimental protocol than for DNA delivery, the capacity of the different LAH4 peptides to deliver mRNA was investigated.

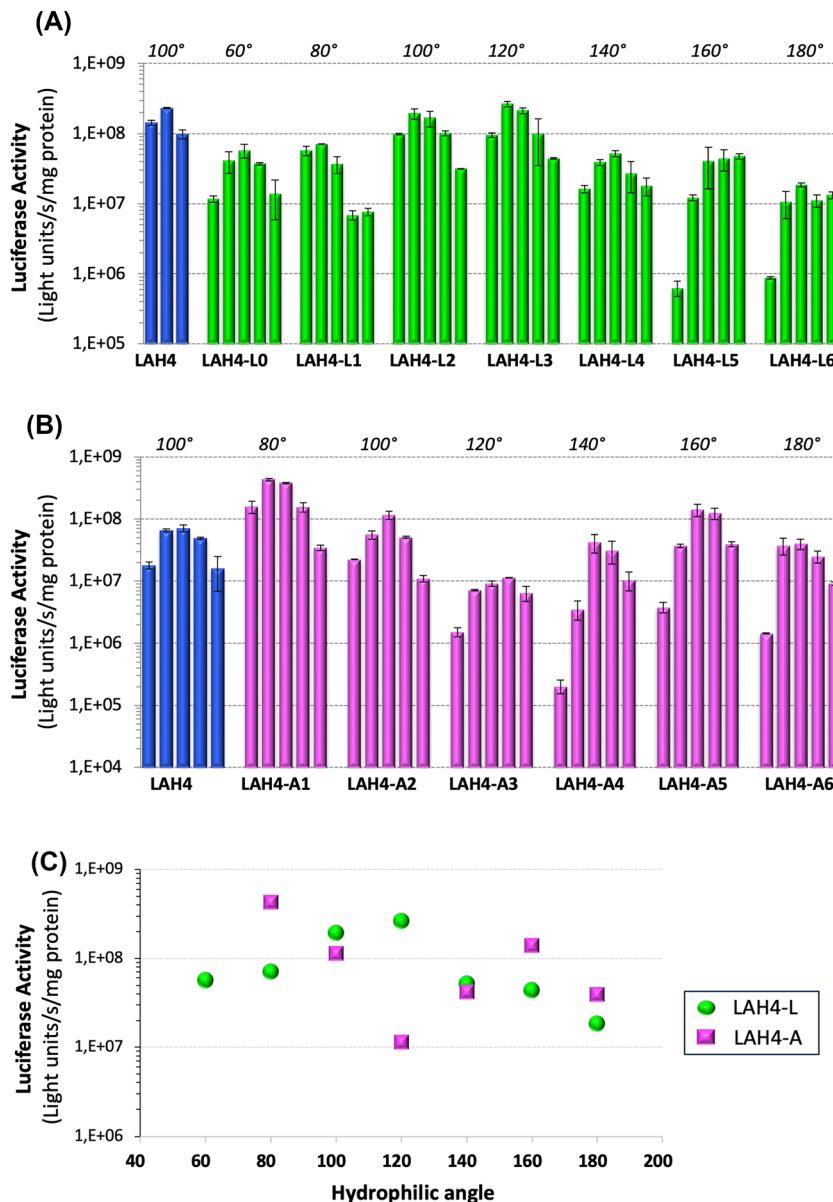
As with DNA, among all the peptides LAH4-A1 was the most efficient one. Globally, the transfection capacity of the different peptides was comparable for DNA and mRNA with the exception of LAH4-L1, which was slightly less efficient for mRNA delivery. When the transfection efficiency is reported as a function of the hydrophilic angle (Figure 1C), we can see that the highest activity is observed for LAH4-A1 and LAH4-L3 for the LAH4-A and L series, respectively, and the activity diminishes when the number of leucines or alanines in the hydrophilic window increases except for LAH4-A5. A very similar trend was previously found for DNA transfection [40].

Notably, the efficacy of the LAH4-A1 in mRNA transfection was confirmed using another cell line, the HCT116 cells (using 150 mM NaCl to generate the complexes). As shown, the LAH4-A1 derivative allowed for higher luciferase expression than LAH4 (see Figures 3 and S3).

3.2 | Design and evaluation of N-terminal modified LAH4-A1 peptides

Linear and branched polyethylenimines of 22–25 kDa are extremely efficient for the delivery of nucleic acids, in particular plasmid DNA [42]. Interestingly, oligoethylenimines (OEIs) (MW < 2000 Da) are significantly less efficient unless they are modified by different types of chemical groups [43–47]. In particular, Chipier et al. found that salicylamide or tyrosine modification of OEIs highly improved their transfection activity, including for mRNA [48]. On the other hand, aromatic tryptophan-substituted OEI proved extremely efficient for DNA delivery [49]. Based on these latter observations, we wondered if grafting tryptophan, tyrosine, or salicylic acid residues onto

FIGURE 1 mRNA transfection of the human glioblastoma cell line U87. The transfection assay was performed in duplicates in 96-well plates using 0.3 μg mRNA luciferase/well. Complexes, generated in 10 mM acetate buffer pH 5, were incubated for 2.5 h in serum-free RPMI medium. Thereafter, the medium was removed and replaced with serum-containing medium. Luciferase activity was measured 24 h post-transfection. The transfection efficiency is expressed as light units/s/mg protein and the reported values are the mean of duplicates. Error bars represent the standard deviation of the mean. The amounts of peptide tested for each peptide for 0.6 μg of mRNA: 2.5, 4, 6, 8, and 12 μg (left to the right). For LAH4 in (A), only three conditions were tested: 4, 6, and 8 μg . In italics are given the hydrophilic angles when peptides are presented in a Schiffer–Edmundson helical wheel projection. To note, the experiments shown in (A) and (B) were done separately, which explains the slight difference in luciferase expression found for LAH4. (C) Representation of the transfection efficiency as a function of the hydrophilic angle. Here, only the conditions giving the highest luciferase expression are reported. Data obtained with LAH4 are not shown in C.



LAH4-A1, the best LAH4 derivative for mRNA delivery, would further increase its transfection efficiency. Therefore, the three new LAH4-A1 shown in Figure 2 were designed and evaluated.

The N-terminal modified peptides were compared to LAH4 and LAH4-A1 for mRNA transfection on HCT116 cells. As the nature of the buffer used to generate the complexes may influence the transfection efficacy, we tested different types of solutions. As expected, LAH4-A1 performed better than LAH4 when the complexes are generated in 150 mM NaCl (Figure 3). Interestingly, while the three new derivatives were less efficient than the parent peptide LAH4-A1 when using NaCl, the transfection efficiency of the tyrosine (noted LAH4-A1-Tyr2) and salicylic acid (noted LAH4-A1-Sali) modified peptides was strongly improved when mRNA complexation occurred in a phosphate buffer pH 6.25 (Figure S3).

Interestingly, whereas PBS, another phosphate buffer, was unable to enhance the efficiency of the new peptides compared to NaCl, a

HBG solution resulted in comparable efficiencies than the phosphate buffer pH 6.25 (Figure S4A,B). This salt dependency of the Tyr2 and salicylic acid derivatives was also observed for DNA transfection (Figure S5).

Next, we wondered how those new CPPs could compare with lipid-based mRNA complexes. Therefore, we compared the transfection efficiency of LAH4-A1 and its two derivatives with the mRNA capabilities of two commonly used cationic lipids, namely, DOTAP and Lipofectamine 2000. The results shown in Figure S6 indicate that the Tyr2 and Sali-derived LAH4-A1 peptides have mRNA transfection capacities that are in same range than those of the two lipid-based formulations.

To try to understand why there is a difference between the complexes generated in 150 mM NaCl and 10 mM phosphate pH 6.25 we made a gel retardation assay using both conditions and subsequently determined the size of the complexes. The mRNA retardation assay

shown in Figure S7 indicates that full retardation is achieved with lower amounts of peptide when complexes are generated in sodium chloride than with the phosphate buffer pH 6.25 (0.5 μ g peptide/1 μ g mRNA in NaCl against 1.5 μ g peptide/1 μ g mRNA in phosphate buffer pH 6.25). Next, the size of the particles in the phosphate buffer pH 6.25 was compared to the one in 150 mM NaCl using dynamic light scattering (DLS). While the complexes LAH4-A1-Tyr2/mRNA had an approximate size of 133 nm when prepared in the phosphate buffer, there size strongly increased in 150 mM NaCl (1352 nm) (Figure S8 and Table 2). Similar results were obtained when DNA was used in place of mRNA (Table 2). These results could suggest that smaller particles are more efficient for transfection when using this LAH4-A1 derivative.

However, a deeper analysis indicates that the size cannot explain all the results. Indeed, the size of the LAH4-A1-Tyr2/mRNA complexes prepared in PBS and in HBG was also measured. The results shown in Table S1 indicates an intermediate size of around 600 nm, which lies between the sizes obtained in 10 mM phosphate pH 6.25 and in 150 mM NaCl. As the complexes generated in HBG allow for a

transfection as efficient as in phosphate buffer while the particles prepared in PBS are less active, we can conclude that a given size of the complexes is not sufficient to confer a high activity to LAH4-A1-Tyr2. This was further confirmed in the subsequent experiments that consisted in measuring the size of LAH4-A1/mRNA, LAH4-A1-Trp2/mRNA, and LAH4-A1-Sali/mRNA complexes in NaCl and phosphate buffer pH 6.25. The results shown in Table S1 indicate that there is no clear relationship between size and the transfection efficiency.

Next, by using a cyanine 5-labeled mRNA, we evaluated whether the cell uptake of the complexes is different when using NaCl or a phosphate buffer pH 6.25. Using LAH4-A1-Tyr2 and LAH4-A1-Sali, we found that cell association/endocytosis is comparable with both buffers. Thus, the increased transfection efficiency observed when using the phosphate buffer pH 6.25 is not due to a difference in association/endocytosis of the CPP/mRNA complexes (Figure S9).

It was previously reported that the acidification process of the endosomal compartment plays a role during LAH4-mediated transfection [23]. Indeed, inhibition of the acidification of the endosomes by inhibitors of the H⁺-ATPase pump decreased the transfection

LAH4-A1-Trp2:

WWKKALLAHALHLLAALALHLLKKA-CONH₂

LAH4-A1-Tyr2:

YYKKALLAHALHLLAALALHLLKKA-CONH₂

LAH4-A1-Salicylic acid:

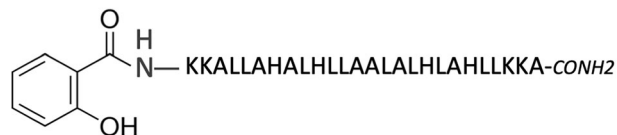


FIGURE 2 Design of three LAH4-A1 derivatives modified at the N-terminus.

TABLE 2 Results of dynamic light scattering (DLS) using LAH4-A1-Tyr2 as complexing agent. The particle size is expressed in intensity.

	mRNA ^a	DNA ^b
Phosphate buffer pH 6.25	133 nm +/- 21.5 (PDI = 0.24)	157 nm +/- 22.5 (PDI = 0.16)
NaCl 150 mM	1352 nm +/- 166 (PDI = 0.25)	1666 nm +/- 152 (PDI = 0.36)

Abbreviation: PDI, polydispersity index.

^aComplexes were generated using 10 μ g LAH4-A1-Tyr2 with 0.8 μ g mRNA luciferase.

^bComplexes were generated using 12 μ g LAH4-A1-Tyr2 with 1.5 μ g DNA luciferase.

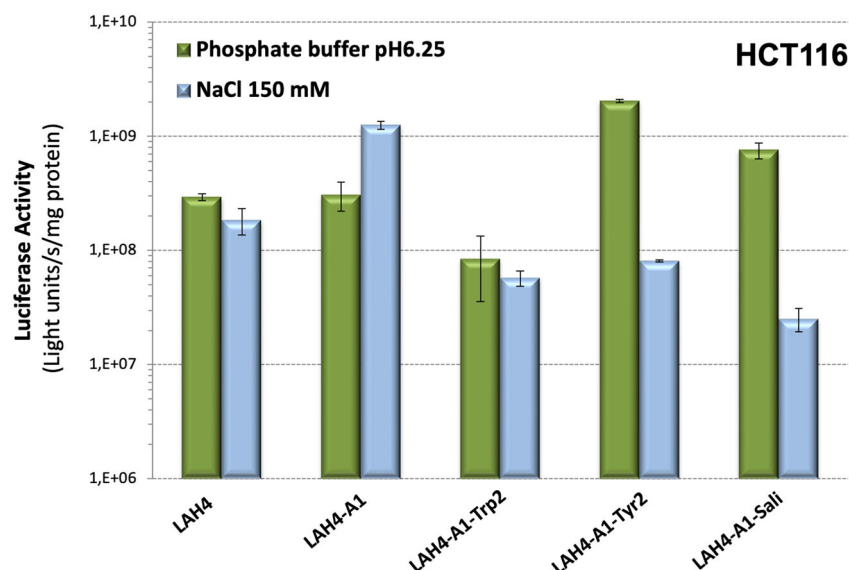


FIGURE 3 mRNA transfection of the human cell line HCT116. The transfection assay was performed in duplicates in 96-well plates using 5 μ g of peptide for complexing 0.5 μ g mRNA luciferase/duplicate. Complexes were generated in either 150 mM NaCl or 10 mM phosphate buffer pH 6.25. The complexes were incubated for 2.5 h in serum-free RPMI medium. Thereafter, the medium was removed and replaced with serum-containing medium. Luciferase activity was measured 24 h post-transfection. The transfection efficiency is expressed as light units/s/mg protein and the reported values are the mean of duplicates (and are shown on a logarithmic scale). Error bars represent the standard deviation of the mean.

efficiency of LAH4. We therefore wondered whether after modification of the N-terminus of the LAH4-A1 peptide the transfection efficiency is also reduced after inhibition of this process. HCT116 cells were transfected in the presence of concanamycin A. As negative control, we chose the monocationic lipid DOTAP because the protonation state of this lipid does not change within the pH range of 4–7. The results show that the efficacy of all the peptides was reduced with concanamycin A, while DOTAP, as expected, was not affected by this treatment (Figure 4). Interestingly, while the transfection efficiency of LAH4 and LAH4-A1 was reduced by six- and fivefold, respectively, it was decreased by 52 and 24 times for the LAH4-A1-Tyr2 and LAH4-A1-Sali peptides, respectively. Thus, the addition of tyrosines or salicylic acid to LAH4-A1 seems to increase the dependence on acidification for endosomal escape.

3.3 | N-terminal modifications of LAH4-A1 limit serum-mediated decrease of transfection

Various studies showed that the presence of serum during the transfection step is deleterious. Therefore, we evaluated the efficiency of the LAH4-A1 derivative in the presence of serum. Whereas transfection of HCT116 cells using LAH4-A1 showed a decrease of efficacy by 500-fold or more in the presence of 10% serum (Figure 5A), with the highest amount of peptide, the luciferase expression was only reduced by 10-fold with the tyrosine derivative and sixfold for salicylic acid modified LAH4-A1. Similar or even better results were

obtained when using the HEK293T for the two modified LAH4-A1 peptides (Figure 5B).

3.4 | Cytotoxicity of the peptide/mRNA complexes

Another important feature for an optimal efficiency of transfection is the cytotoxicity of the particles used for transfection. Therefore, the toxicity of the different formulations was evaluated by using an MTS assay on HCT116 cells. This cell proliferation assay, which measures the metabolic activity of the cells, is based on the reduction of the MTS tetrazolium compound by viable cells to generate a colored dye. As shown in Figure 6, for all conditions, the cell viability was higher in the presence of serum as compared to the serum-free condition. Without serum, the three CPPs induced up to 60% decrease of the metabolic activity with the salicylic acid derivative being the one with the highest toxicity. However, in the presence of 10% serum, the cell viability with the two LAH4-A1 derivatives was of 100% (Figure 6).

To note, using a CellTiter-Glo 2.0 cell viability assay that is based on cell metabolic activity by quantification of ATP released after cell lysis in the culture medium, we confirmed the MTS results, namely, that in the absence of serum the cytotoxicity is higher and that LAH4-A1-Sali is the CPP that induces the highest cytotoxicity (Figure S10A). The cytotoxicity of the CPPs in the absence of mRNA was also evaluated and as illustrated in Figure S10B, it was low for the three peptides, even in the absence of serum.

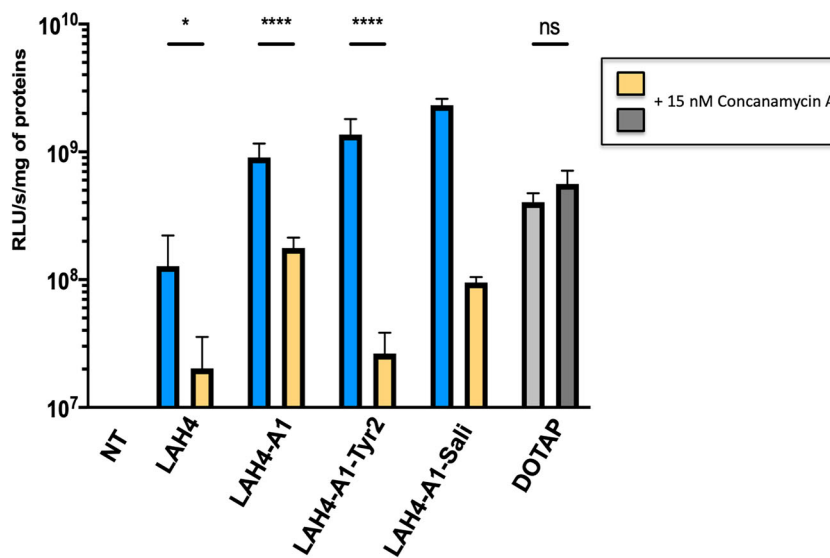


FIGURE 4 HCT116 cell transfection in the presence of a proton pump inhibitor. The transfection assay was performed in triplicates in 96-well plates using 1.75 μg of CPP for complexing 0.25 μg mRNA luciferase/well. mRNA complexes were generated in 150 mM NaCl for LAH4 and LAH4-A1 and in phosphate buffer pH 6.25 for LAH4-A1-Tyr2 and LAH4-A1-salicylic acid. The cationic lipid DOTAP was used at an N/P of 3. Complexes were incubated for 2.5 h in serum-free RPMI medium in the presence or not of 15 nM of concanamycin A. Thereafter, the medium was removed and replaced with serum-containing medium for the next 24 h before luciferase activity measurement. Transfection efficiency is expressed as light units/s/mg of proteins. Reported values are the mean of $n = 2$ independent experiments (each done in triplicates) except for LAH4-A1-Sali for which $n = 1$. Error bars represent one SD. The statistics (not done for the LAH4-A1-Sali) represent a one-way ANOVA test between conditions with or without concanamycin A. Distribution normality was confirmed using a Shapiro–Wilk test. ns = $P > 0.12$; * = $P \leq 0.03$; **** = $P \leq 0.0001$.

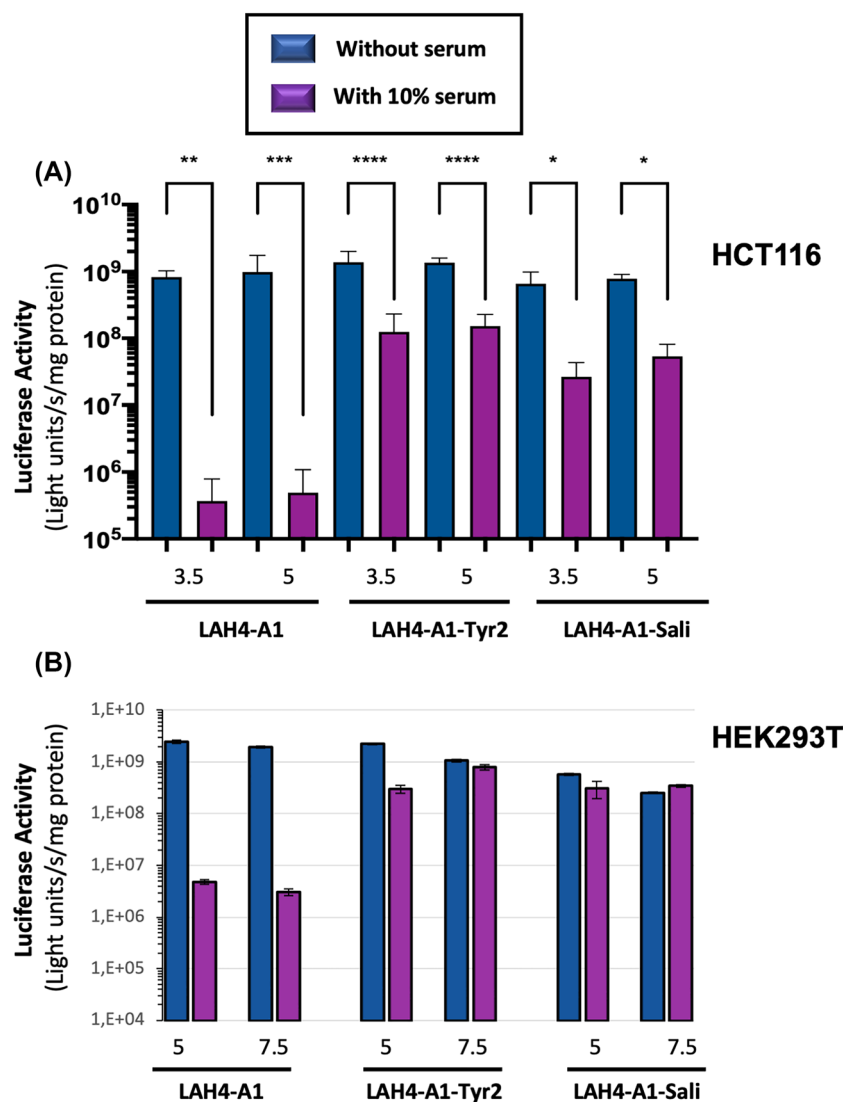


FIGURE 5 mRNA transfection of HCT116 (A) and HEK293T (B) cells in the presence or absence of serum. The transfection assays were performed in duplicates in 96-well plates using the indicated amounts of peptide for complexing 0.5 μ g mRNA luciferase/duplicate. mRNA complexes were generated in 150 mM NaCl for LAH4-A1 and in phosphate buffer pH 6.25 for the two LAH4-A1 derivatives. The complexes were incubated for 2.5 either in serum-free RPMI medium or in culture medium with 10% serum. Thereafter, the medium was removed and replaced with serum-containing medium. Luciferase activity was measured 24 h post-transfection. The transfection efficiency is expressed as light units/s/mg protein, and the reported values are the mean of duplicates (blue and purple bars). Error bars represent the standard deviation of the mean. The statistics indicated in (A) represent a one-way ANOVA test between conditions with or without serum. Distribution normality was confirmed using a Shapiro–Wilk test. * = $P \leq 0.03$; ** = $P \leq 0.002$; *** = $P \leq 0.0002$; **** = $P \leq 0.0001$.

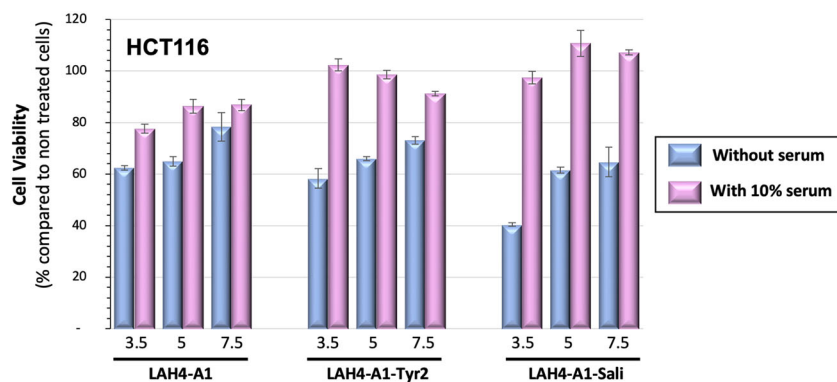


FIGURE 6 Evaluation of the cell viability after transfection of HCT116 cells. The MTS assay was performed in duplicates in 96-well plates using the indicated amounts of peptide for complexing 0.5 μ g mRNA luciferase/duplicate. mRNA complexes were generated in 150 mM NaCl for LAH4-A1 and in phosphate buffer pH 6.25 for the two LAH4-A1 derivatives. The cells were transfected in serum-free or serum containing (10%) medium for 2.5 h, and then medium was replaced with fresh one containing serum. The cells were incubated for additional 21.5 h at 37°C, and then the MTS assay was done. The 100% cell viability was obtained using untreated cells (cells without serum during the 2.5 h incubation step for the blue bars and with 10% serum for the pink bars).

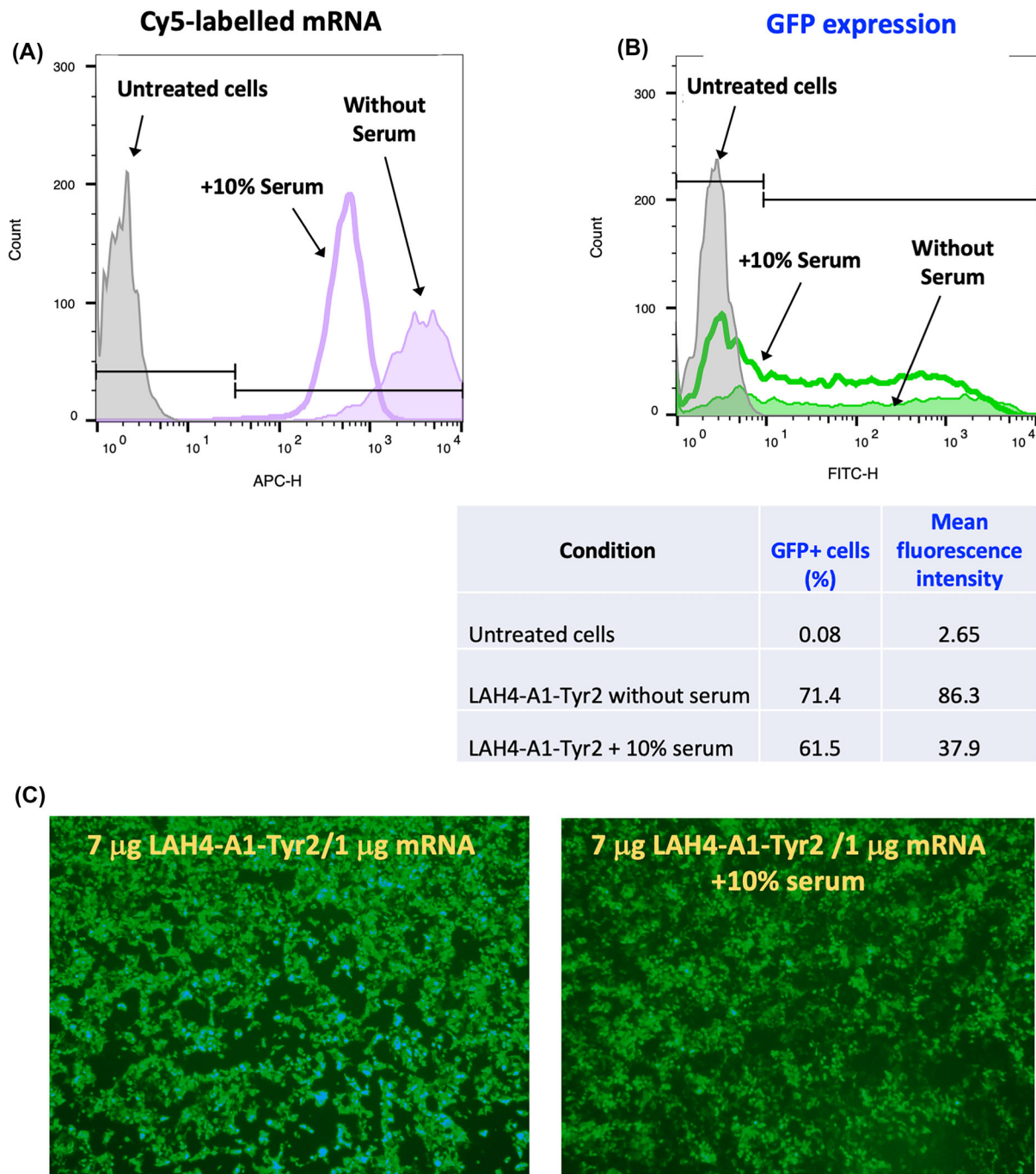


FIGURE 7 mRNA transfection of HEK293T cells in the presence and absence of serum. HEK293T cells plated in 48-well plates were treated as follows: The complexes were generated in phosphate buffer pH 6.25 with 7 μ g LAH4-A1-Tyr2/1 μ g mRNA/well. For (A) we used an mRNA Luc labeled with Cy5 while for (B,C) an mRNA expressing GFP was used. The complexes were incubated for 2.5 h either in serum-free RPMI medium or in culture medium with 10% serum. (A) After 2.5 h of incubation at 37°C, medium was removed, and cells were washed and harvested and finally used for flow cytometry analysis. (B,C) After the incubation, the medium was removed and replaced with serum-containing medium. GFP expression was measured 24 h post-transfection using flow cytometry (B) and fluorescence microscopy (C). (A and B) In gray, the fluorescence of untreated cells; the bold line shows the fluorescence obtained in the presence of 10% serum; the green and purple area show the fluorescence obtained using LAH4-A1-Tyr2/mRNA complexes in the absence of serum. To note, in experiment (B) for the serum-free condition, we analyzed 3500 events while for the condition with serum, 8900 events were counted.

As the LAH4-A1 tyrosine derivative (LAH4-A1-Tyr2) seemed to be more interesting than the salicylic acid one (less cytotoxic while being slightly more efficient), we focused on this CPP in the next study.

3.5 | LAH4-A1-Tyr2-mediated GFP expression

To go further in the evaluation of the LAH4-A1-Tyr2 efficiency in mRNA transfection, we evaluated the amount of nucleic acid that is

associated/delivered in the cell by the tyrosine-modified peptide. For this, HEK293T cells were transfected with an mRNA labeled with the fluorophore Cy5 associated to LAH4-A1-Tyr2 with or without serum. In both cases, 100% of cells are Cy5 positive, showing that the mRNA-peptide complexes have reached all the cells. However, in presence of serum, a lower level of fluorescence was detected, suggesting that less complexes associated with/entered into the HEK293T cells (Figure 7A).

To ensure that the mRNA was (i) delivered into the cell (and not just associated to the cell membrane) and (ii) translated in proteins, the percentage of cell transfected with an mRNA encoding for the green fluorescent protein (GFP) was evaluated on HEK293T 24 h post-transfection in flow cytometry and microscopy. As shown in Figure 7B, in the presence of serum, 61.5% of cells were GFP+ (Figure 7B) showing the functionality of the delivered mRNA. The absence of serum only slightly increased the percentage of positive cells (71%). To note, in the presence of serum, the mean fluorescence intensity (MFI) was lower as shown by flow cytometry and confirmed by fluorescence microscopy (Figure 7B,C).

LAH4-A1-Tyr2 finally proved also able to transfect very efficiently the human immortalized keratinocytes HaCaT with a percentage of GFP-positive cells above 90% (Figure S11).

4 | DISCUSSION

We have previously shown that CPPs from the LAH4 family have different properties, among which the capacity to deliver several types of cargo into mammalian cells [37, 40]. Until now, we mostly focused on their DNA transfection capabilities. In the present manuscript, we show that LAH4 and some of its isomers, in particular LAH4-A1, could also efficiently deliver mRNA into cells. As previous studies reported that modification of OELs by salicylamide or tyrosines improved their transfection activity, including for mRNA [41], we decided to evaluate three LAH4-A1 derivatives modified at the N-terminus by either tryptophans, tyrosines, or salicylic acid. The results showed that the Tyr2 and the Sali derivatives, but not the tryptophan-modified peptide, had very good DNA and mRNA transfection properties when the complexes were generated in a phosphate buffer pH 6.25 (or HBG). Such a salt/buffer-dependent activity although unexpected is not unique as it has also been observed, for example, with lipospermine/DNA complexes [50] and poloxamers [51, 52]. We also found that the complexes generated in the phosphate buffer pH 6.25 were approx. 10 times smaller than those generated in 150 mM NaCl. Thus, as already observed with LAH4 [24], with the recently described HELP peptide [18], and with CPPs [21, 22], salt-rich conditions such as 150 mM NaCl increase the size of the complexes. However, sodium chloride not only induces aggregation but also negatively affects the transfection efficiency of the tyrosine-modified peptide. This is in contrast to what has been observed with the HELP, for example, for which the smaller complexes and the larger ones had almost comparable transfection efficiencies [18].

More importantly, we found that while the mRNA transfection activity of LAH4-A1 was highly serum sensitive—with a reduction in luciferase expression of more than 2 log—it was significantly less the case for the Tyr2 and Sali derivatives. Finally, the flow cytometry results using a Cy5-labeled mRNA showed that while all the cells take up the LAH4-A1-Tyr2 complexes, only 60%–70% express GFP. This suggests that in 30%–40% of cells, the mRNA did not reach (in sufficient amounts) the cytosol. Thus, although the new CPPs developed here demonstrated robust transfection efficiencies, there is still the need for further improvements. However, our findings open new perspectives for the development of serum resistant LAH4-based CPPs for mRNA delivery.

AUTHOR CONTRIBUTIONS

Candice Dussouillez, Morane Lointier, and Mohammed-Karim Sebane performed the experiments; Sylvie Fournel and Burkhard Bechinger contributed to the design of the study; Antoine Kichler designed and supervised the study and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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