WRAP-based nanoparticles for siRNA delivery: A SAR study and a comparison with lipid-based transfection reagents.

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Research

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Abstract

Recently, we designed novel amphipathic cell-penetrating peptides, called WRAP, able to transfer efficiently siRNA molecules into cells. In order to gain more information about the relationship between amino acid composition, nanoparticle formation and cellular internalization of these peptides composed of only three amino acids (leucine, arginine and tryptophan), we perform a structure activity relationship (SAR) study. First, we compared our WRAP1 and WRAP5 peptides with the C6M1 peptide also composed of the same three amino acids and showing similar behaviors in siRNA transfection. Afterwards, to further define the main determinants in the WRAP activity, we synthesized 13 new WRAP analogues harboring different modifications like the number and location of leucine and arginine residues, the relative location of tryptophan residues, as well as the role of the α-helix formation upon proline insertions within the native WRAP sequence. After having compare the ability of these peptides to form peptide-based nanoparticles (PBNs) or not using different biophysical methods (circular dichroism, dynamic light scattering, gel shift assay) and to induce a targeted gene silencing (luciferase assay) in cells, we were able to establish the main sequential requirements of the amino acid composition of the WRAP peptides to maintain a good siRNA transfection efficacy. In addition, upon measuring the WRAP-based siRNA transfection ability into cells compared to several non-peptide transfection agents available on the markets, we confirmed that WRAP peptides induced an equivalent level of targeted gene silencing but in most of the cases with significant lower cell toxicity as clearly shown in clonogenic assays.

Introduction

Transfection of siRNAs is nowadays widely employed to specifically knock-down the cellular expression of any targeted protein. However, siRNAs are unable to translocate within the cells without the use of transfection reagents. After the development of various compounds such as cationic lipids, polymeric molecules or inorganic nanoparticles to promote their cell delivery, peptides called cell-penetrating peptides (CPPs) have been also designed and used alternatively. During the last years, several parameters have been investigated to understand how CPPs could be more efficient in transferring into cells different types of nucleic acids (plasmids, siRNAs, etc) [1, 2]. Therefore, different substitutions, deletions and modifications within their primary sequence have been performed and the translocating activities of the corresponding peptides have been compared to the parental CPPs. These investigations included the number of arginine residues [3], the presence and the location of tryptophan residues within the sequence [4, 5], the integration of apolar moieties [6, 7] either as non-natural amino-acids [8], as fatty acids [9, 10] or as hydrophobic groups directly grafted onto the CPP [11–14]. Moreover, structural studies have been also conducted to define whether the CPP secondary structure could influence the translocating process [15].

In our seminal paper highlighting the potential of tryptophan (W)- and arginine (R)-rich Amphipathic Peptides (WRAP) [16], we noticed already that the hydrophobic character of the CPP was crucial, since leucine replacements by less hydrophobic alanine or glycine residues decreased dramatically the ability of siRNA complexation and of peptide-based nanoparticle (PBN) self-assembling. In line with this,
colloidal characteristics performed by Dynamic Light Scattering (DLS) indicated that these side-chain replacements (Leu◊Ala or Leu◊Gly) induced the formation of aggregates (> 1,000 nm of diameter) [16]. Therefore, the overall hydrophobicity of the CPP appeared to influence the way of PBN self-assembling, probably governing the size of the formed nanoparticles [17–19].

Others groups also investigated the effect of coupling hydrophobic moieties to the peptide moieties in order to promote the siRNA delivery. These include the work of Neuberg and co-workers who coupled either a simple amino acid such as histidine or dipeptides to a dioctadecylamine tail through a hydrophilic linker [20]. They evidenced better vectors for siRNA transfection upon this hydrophobization. More intriguingly, Mozaffari and colleagues reported that the simple addition of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) to different peptide:siRNA nanoparticles appeared sufficient to enhance significantly the silencing efficacy of siRNA complexed with a cyclic CPP made only of tryptophan and arginine residues [21]. In this case, there was no direct chemical grafting, but only a simple mix of the DOPE to the formed nanoparticles.

It has been referred in several reports that amphipathic helical peptides composed of hydrophobic and cationic amino acids exposed on different sides of the helix could be typical CPPs [22]. Chen's group designed a peptide, C6M1, which showed a random/helical structure in water with the ability to attain a helical conformation upon interactions with anionic components or membrane-mimicking environments [23]. We also demonstrated with the CADY-K peptide by biophysical methods that α-helix was an important prerequisite for stable PBN formation [24]. Both peptides have been shown to vectorize efficiently siRNAs in cells. Wada and colleagues designed amphipathic helical 12-mer peptides containing α,α-disubstituted α-amino acids known to stabilize peptide secondary structures [25]. But in this case, it is worth noticing that the hydrophobic character has been slightly modified upon the grafting of α-aminoisobutyric acid (Aib) moieties, each containing two methyl groups. Therefore, it might be difficult to distinguish which of the helicoidal structure or the hydrophobic character triggered the improved siRNAs transfection.

It is widely admitted that the inability of nucleic acids to enter cell mainly relied on its anionic nature which has to interfere with the negatively charged glycoproteins of the cell membrane and with the highly anionic nature of the lipid bilayer while entering the cell and the cytoplasm. There is a clear evidence that cationic amino acids within the CPP sequence were of high importance in the formation of PBNs with negatively charged oligonucleotides such as plasmids or siRNAs. We and others notified a well-defined stoichiometry between the CPPs and the nucleic acids to ensure a full complexation, leading in general to a positive zeta potential of the PBNs [16, 23, 26]. Indeed, a positive zeta potential of PBNs was expected to favor interactions with the anionic cell surface components. The charge ratio between peptides made only of cationic lysine residues over anionic charges of nucleic acids was also shown to be critical parameters for forming stable particles with plasmid DNAs and siRNAs [27]. In fact, without this initial and crucial packaging step, delivery and transfection cannot be achieved subsequently. These ionic interactions between CPPs and nucleic acids in general, but more particularly with siRNAs, could be displaced or disrupted by other polyanionic moieties such as heparin sulfate for instance [28]. This
displacement or disruption of these ionic interactions could reflect the way PBNs are destabilized to release siRNA within the cells. Therefore, the cationic nature of the CPPs appeared crucial to form but also to destabilize nucleic acid-loaded PBNs. There are three natural cationic amino acids available (arginine, lysine and histidine) to interact with the anionic charges of nucleic acids and all three have been in depth investigated to define their relative influence on the translocating activity. For instance, it has been postulated and demonstrated that histidine residues played a role in the “proton sponge” effect thanks to the imidazole group present in its side chain [29]. Histidine residues were able to capture protons entering the endosome, then leading to the swelling of the endosomes and triggering its membrane disruption, thus allowing the nucleic acid to enter the cytoplasm and to diffuse within the cell. This disruption of the endosomal membrane is indeed expected and strongly desired for PBNs entering the cells through any of the endocytic routes. Since the internalization pathway of our series of WRAP peptides appeared to be mainly a direct translocation through the cell membrane, as recently demonstrated by our group [30], no benefit should be expected from an histidine residue integration within our series of peptides.

Along this line the literature abounds in demonstrations of the key role played by arginine residues compared to lysine residues in the translocating properties of CPPs either used alone or associated with cargo molecules. This has been the case following deletion of arginine residues within the Tat peptide, one of the first CPPs to be discovered [31]. Few years later, efficient CPPs only made of arginine residues have been shown to be internalized when they were made of 6–10 arginine residues only [32], thus highlighting the interest of integrating arginine residues in CPP primary sequences. It also appeared undisputable that glycosaminoglycans (GAGs) but also phospholipidic heads exposed at the cell membrane surface played a role in the cellular uptake of arginine-rich CPPs. Bidendate interactions between the guanidinium groups of arginine and phosphate groups of phospholipids or sulfate groups of GAGs have been shown to be responsible of these interactions [33]. It has been also quantified that the substitution of arginine residues by lysine, ornithine or histidine residues within CPP sequences reduced dramatically their ability to enter cells [34]. Consequently, we integrated in these peptides only arginine residues as cationic groups.

Another amino acid which appeared to be important for promoting the translocation properties of these CPPs was the tryptophan residue [35]. This has been highlighted in a study where a tryptophan residue was replaced by a tyrosine leading to the loss of cellular transfection of siRNAs despite the formation of much smaller nanoparticles [20]. The importance of tryptophan residues has been also confirmed in a recent publication showing the proper internalization of a Penetratin analogue only composed of arginine and tryptophan [36]. Interestingly, a corresponding 9-mer peptide made only with arginine and leucine was not taken up by cells, thus already confirming the peculiar role of the tryptophan residue (see below). However, we cannot exclude that the behavior of these peptides, used without any cargo molecules, could be altered or modified upon their binding to nucleic acids for instance. We also showed the importance of tryptophan residues since reducing down to two residues the number of tryptophan in a CPP sequence could directly impair PBNs formation (aggregation over 1,000 nm of diameter), and consequently, their cell translocation activity [16].
In 2013, Jafari and co-workers introduced a 18-mer amphipathic peptide C6 designed for siRNA delivery, but mainly suffering of a poor solubility [23]. Therefore, three leucine residues were replaced by three tryptophan residues leading to the C6M1 peptide. As mentioned above, this peptide composed only of arginine, leucine and tryptophan residues presented a random/helical structure in water with the ability to attain a helical conformation in the presence of anionic components (nucleic acids or membrane-mimicking compounds) [23, 37]. These characteristics of conformational changes upon complexation with siRNAs were also observed for other amphipathic CPPs such as CADY-K [24], RICK [17] or our recently designed WRAP peptides [16, 30].

This latter series of WRAP peptides was also made of tryptophan, arginine and leucine residues only, and was indeed close to the amino acids composition of the C6M1 peptide. In our quest to further depict the role of these arginine, leucine and tryptophan amino acids with the WRAP sequences, we wished to perform an in-depth structure-activity relationship study (SAR study) and to compare the behavior of these peptides upon various sequence modifications both in their ability to form siRNA-loaded nanoparticles, and more importantly, in their capacity to induce a biological silencing response as assessment of an effective and unambiguous intracellular delivery of siRNAs. We thus defined the importance or not of different features within our WRAP peptide sequences which could be essential to understand the high siRNAs transfection potential of our PBNs.

Finally, since the main use of such transfecting peptides is motivated by the cellular transport of nucleic acids, either plasmids or siRNAs, we wished to evaluate the efficacy and the toxicity of our WRAP peptides series compared to the main siRNA transfecting agents available on the market.

**Results**

*Comparison of WRAP and C6M1: two CPPs with a close amino acid composition.*

To get more insight on the role of amino acid composition during the transfection of siRNAs, we compared WRAP1 (W1) and WRAP5 (W5) with the C6M1 peptide developed by Chen’s group [23,37]. Additionally, we implemented the C6M1-L peptide resulting from a synthesis deletion. These four peptides have nearly the same amount of tryptophan residues (three or four depending on the peptide) but differ more importantly in the amount of arginine (four for W5 and W1 and seven for C6M1) or leucine residues (six to eight depending on the peptide). All together these resulted in longer peptides in combination with a higher number of charges for C6M1/C6M1-L compared to the WRAP series (Figure 1A).

By measuring their biological activity using a human glioblastoma U87 cell line stably transfected for constitutive expression of FLuc/NLuc reporter genes, we observed for both peptides C6M1 and C6M1-L a lower silencing activity (60% and 35%) compared to both WRAP peptides (80%-90%) encapsulating in each case the same amount of siRNA (10 nM) (Figure 1B). Considering the close similarity in amino acids content between the two families of peptides and differences in the primary sequence, this result suggested that the amount of leucine and arginine residues were important for the silencing activity of the resulting PBNs. This could be the consequence of the higher number of arginine in the C6M1 series...
compared to the WRAP series (4). Therefore, we could formulate objectively the hypothesis that a higher content of arginine is unfavorable for a strong biological response. Indeed, in our biological assay, we measured a global effect, ranging from the PBNs formation till the siRNA effects at the RISC system. In between, the PBNs have to internalize the cells and siRNAs have to be properly decaged from the PBNs. A lower decaging for the C6M1 PBNs could explain a weaker release and consequently a lower biological response of siRNA molecules because of the higher content of ionic interactions. In line with this, we clearly observed the direct influence of arginine residues in the decaging rate of siRNAs from PBNs made of WRAP peptides with different amount of arginine residues (see below).

Another noticeable difference was the lower number in leucine residue for the C6M1 peptide series (6 or 7) compared to the WRAP series (8). A lower number of leucine residues seemed to correlate with a lower biological response. Along this line, we observed that the deletion of one leucine in the C6M1-L peptide compared to the native C6M1 peptide seemed to further reduce the silencing property of the PBNs (Figure 1B). Therefore, we investigated whether the N- and C-terminal and internal leucine doublets in our WRAP peptide series were indispensable all along the different steps from the nanoparticle formation towards the recorded gene silencing activity.

**Dissecting the role of the leucine doublets within the WRAP sequence.**

To confirm this hypothesis, we synthesized analogues deleted of one leucine located at both N- and C-terminal regions of the WRAP peptides (W1-2L and W5-2L, respectively). Additionally, we synthesized the W5-2Lm peptide to analyze the role of the intra-sequential leucine doublets and the W5-4L peptide with all possible leucine doublet deletions (Table 1).

Both parental peptides, W1 and W5, were compared to their respective DLeu-analogues by agarose shift assay in order to verify first their ability to complex siRNAs (Figure 2A and 2B). Without the peptide addition, siRNAs migrated into the agarose gel (= 100% signal). But when complexed with an increased molar ratio of W1 and W5 peptides, siRNA migration was prevented in a molar ratio-dependent manner as previously reported [16] (Figure 2A and 2B). W1-2L, W5-2L, W5-2Lm and W5-4L clearly complexed siRNAs similarly to the parental peptides, with a complex formation starting at a molar ratio of 10 (R = 10 for >50% of complexed siRNAs). However, we decided to use the molar ratio R = 20 for PBN formulations since we showed that PBNs were thus more stable [16,17,24].

Additionally, biological assays were performed using the same PBN solutions studied by CD and DLS experiments. This allowed us to directly correlate the PBN formation with the biological activity, thus avoiding potential artifacts due to the preparation of different formulations.
As reported previously [16], WRAP peptide solutions were nearly unstructured on their own (W1 = random coiled and W5 = turn conformation). They adopted an α-helical conformation once incubated with siRNAs. Circular dichroism (CD) measurements were performed for all peptide analogues, both alone and in the presence of siRNAs to verify whether leucine doublet deletions would influence any conformational switch (Figures 2C and 2D and Additional files 1 and 2). W1-2L revealed a conformational switch showing the tendency of a helical structure formation (increased maxima at 195 nm and induced minima at 202 nm or 227 nm) (Figure 2C). In contrast, W5-2L, W5-2Lm and W5-4L remained mainly unstructured with the presence of the two minima (203 and 227 nm) suggesting a turn conformation as for W5 (Additional file 2 A-D). Upon incubation with siRNAs, the tryptophan cluster contribution, corresponding to the minima around 227 nm, was maintained for all mutants but the lack of leucine gave rise to a small maximum at 210 nm (Figure 2D). To better understand these differences, we performed in silico 3D structure prediction of WRAP and analogue peptides alone (Figure 2E). After computation, the peptide models generated by PEPstrMOD [38,39] in a hydrophilic environment revealed that W1, W5, W1-2L were able to adopt α-helical structure. W5-2Lm and W5-4L had no helical structure, confirming the CD measurements. It is noteworthy to mention that the leucine-deleted peptides W5-2L was predicted to adopt a short helix, which is in contradiction to the CD evaluation. However, in case of discrepancies

### Table 1: Characterization of the WRAP peptides and their analogues used in this study.

| ID       | Sequences                  | nb AA | charges | Z-ave (nm) | Pdi     | Stability | Helix (CD) | PEPstrMOD | Luc silencing | % KD   | @10 nM siRNA |
|----------|----------------------------|-------|---------|------------|---------|-----------|------------|------------|--------------|--------|---------------|
| Parental peptides |                            |       |         |            |         |           |            |            |              |        |               |
| W1       | LLKKKRRLLLWLLWLLWLLWLLWLL | 10    | +5      | 91 ± 25   | 0.34 ± 0.05 | YES       | YES       | 12         | 5.3         | YES | 91            |
| W5       | LLKKKRRLLLWLLWLLWLLWLLWLL | 15    | +5      | 81 ± 29   | 0.28 ± 0.02 | YES       | YES       | 10         | 2.8         | YES | 88            |
| AL analogues |                            |       |         |            |         |           |            |            |              |        |               |
| W1-2L    | LLKKKRRLLLWLLWLLWLLWLLWLL | 14    | +5      | 111 ± 9   | 0.42 ± 0.18 | NO        | YES       | 9          | 2.5         | YES | 80            |
| W1-Lm    | LLKKKRRLLLWLLWLLWLLWLLWLL | 15    | +5      | 127 ± 7   | 0.36 ± 0.04 | NO        | NO        | 8          | 2.2         | YES | 63            |
| W5-2L    | LLKKKRRLLLWLLWLLWLLWLLWLL | 15    | +5      | >1000     | 0.22 ± 0.04 | NO        | NO        | 7          | 1.9         | NO | 11            |
| W5-2Lm   | LLKKKRRLLLWLLWLLWLLWLLWLL | 13    | +5      | >1000     | 0.22 ± 0.01 | NO        | NO        | 0          | 0.0         | NO | 11            |
| W5-4L    | LLKKKRRLLLWLLWLLWLLWLLWLL | 11    | +5      | >1000     | 0.28 ± 0.03 | NO        | NO        | 0          | 0.0         | NO | 3             |
| R analogues |                            |       |         |            |         |           |            |            |              |        |               |
| W1-4R    | LLKKKRRLLLWLLWLLWLLWLLWLL | 13    | +5      | 441 ± 175 | 0.50 ± 0.05 | NO        | YES       | 7          | 1.9         | NO | 0             |
| W1-6R    | LLKKKRRLLLWLLWLLWLLWLLWLL | 18    | +7      | 111 ± 55  | 0.40 ± 0.08 | YES       | YES       | 14         | 5.9         | YES | 79            |
| W5-4R    | LLKKKRRLLLWLLWLLWLLWLLWLL | 15    | +5      | 118 ± 93  | 0.41 ± 0.11 | YES       | YES       | 8          | 2.2         | YES | 64            |
| W5-6R    | LLKKKRRLLLWLLWLLWLLWLLWLL | 17    | +7      | 64 ± 9    | 0.34 ± 0.07 | YES       | YES       | 11         | 3.1         | YES | 57            |
| P analogues |                            |       |         |            |         |           |            |            |              |        |               |
| W1-1P    | LLKKKRRLLLWLLWLLWLLWLLWLL | 17    | +5      | 151 ± 28  | 0.35 ± 0.02 | NO        | NO        | 5          | 1.4         | YES | 37            |
| W5-2P    | LLKKKRRLLLWLLWLLWLLWLLWLL | 17    | +5      | 242 ± 60  | 0.37 ± 0.01 | NO        | NO        | 6          | 1.7         | NO | 10            |
| W-mix analogues |                        |       |         |            |         |           |            |            |              |        |               |
| W1-MMix  | LLKKKRRLLLWLLWLLWLLWLLWLL | 16    | +5      | 102 ± 33  | 0.36 ± 0.04 | YES       | YES       | 15         | 3.6         | YES | 80            |
| W5-MMix  | LLKKKRRLLLWLLWLLWLLWLLWLL | 15    | +5      | 94 ± 35   | 0.36 ± 0.04 | NO        | NO        | 11         | 3.1         | YES | 41            |

Footnotes: Represented in the table are the primary sequences (red = amino acids implicated in the helix), the number of amino acids (AA), the number of charges, the mean size [Z-average, nm] and the polydispersity index (Pdi), the stability of the PBNs after a 4 day-period at 4°C, the helix signal in CD measurements, the number of amino acids implemented in the helix (AA in helix), and the calculated number of helix turn (number of amino acids in the helix divided by 3.6 as predicted by PEPstrMOD calculation), the luciferase silencing activity and the level of silencing in percentage at the dose of 10 nM siRNA.
between the measured and the expected results about the helical content within the peptides, it is worth noticing that we were more confident with the results provided experimentally by CD measurements than with those predicted by a theoretical modelling system.

Afterwards, colloidal features of WRAP:siRNA complexes (R = 20) were characterized by Dynamic Light Scattering (DLS) to determine the nanoparticle size and polydispersity of size distribution. Intensity measurements (%) revealed that W1 and W5 formed PBNs with diameters of 80-100 nm with polydispersity indexes (PdI) around 0.3 (Table 1) as reported previously [16]. A comparable result could be observed for W1-2L, showing a mean size of 111 nm with a PdI of 0.42. In contrast, W5-2L, W5-2Lm and W5-4L showed mean sizes higher than 1,000 nm indicating that these three peptides were not able to form PBNs in the presence of siRNAs.

**Evaluating the role of arginine residues within the WRAP sequence.**

Since the C6M1 peptide harbored arginine residues at both extremities, we wanted to evaluate the effect of arginine residue addition at both N- and C-terminal ends of the WRAP sequences by synthesizing analogues with one additional arginine residues at both ends of the peptide (Arg = 6 for W1-6R and W5-6R). However, because the comparison between C6M1 and WRAP peptides suggested the importance to keep an identical amount of arginine residues in order to maintain a good biological effect (Figure 1B), we designed peptides with two additional arginine residues at both extremities, but with two arginine residues less within the sequence to keep the same charge number as the parental peptides (Arg = 4 for W1, W5, W1-4R and W5-4R) (Table 1).

As performed above for the DLeu-analogues, we first evaluated by agarose gel shift assay the ability of these Arg-analogues to form PBNs in the presence of siRNAs depending on the used molar ratio required for an optimal siRNA complexation (Figure 3A). Increasing the number of arginine residues clearly improved complex formation compared to the parental peptides (molar ratio R = 7.5 for W1-6R and W5-6R versus R = 10 for W1 and W5, respectively). This was likely related with the higher number of positive charges in peptides containing 6 arginine residues. In contrast, the simultaneous N-and C-terminal arginine addition together with the internal arginine deletion seemed to slightly impact negatively nanoparticles formation as revealed by the higher molar ratio required to fully complex the siRNA (R = 12.5 for W1-4R and W5-4R). This could be related with the lower biological effect observed for the C6M1 peptides compared to the WRAP peptides (Figure 1B) and to the hypothesis of a weaker decaging ability for peptides containing a higher number of arginine residues. On the other hand, the CD spectra showed in both cases that W1-6R and W5-6R adopted in the presence of siRNAs a helical conformation comparable to those recorded with the parental peptides (Figure 3C and 3D). For the W1-4R siRNA-loaded complexes, we observed some slight changes in the helical structure while the lack of arginine inner residues of W5-4R do not give any conformational change compared to W5 in the presence of siRNA. Structure predictions by PEPstrMOD [38,39] in a hydrophilic environment revealed that the potential helix
was shorter for W1-4R and W5-4R compared to the other peptides (Figure 3E). These behaviors could indeed impair with the capacity of these peptides to form stable PBNs with the siRNA.

As expected from the results above, all Arg-analogues complexed with siRNAs formed PBNs with mean sizes in the 100 nm range as measured by DLS (Table 1). However, we observed for W1-4R slightly bigger (441 ± 175 nm) and more dispersed PBNs (0.50 ± 0.05).

In conclusion, adding two arginine residues to the N- and C-terminal end of WRAP sequences seems to induce PBN formation at lower molar ratio without perturbing importantly the size of the formed PBNs. Displacing the two internal arginine residues at the N- and C-terminal end of WRAP sequences (W1-4R and W5-4R - same number of arginine residues as the parental peptides) resulted in minimal conformational changes and PBN formation, with the exception of W1-4R which formed four times bigger PBNs.

Cellular activity of the different WRAP PBNs was evaluated on a luciferase positive human glioblastoma U87 cell line. To this aim, PBNs solutions used for CD and DLS measurements were diluted to siRNA concentrations of 5 nM, 10 nM and 20 nM and directly applied on cells to perform the luciferase assay. W1:siRNA and W5:siRNA gave impressive silencing activities for the three siRNA concentrations (Figures 4A, 4B and Table 1). We noticed that these inhibitions were higher than inhibition levels previously reported [16]. For instance, we obtained before around 60% luciferase activity remaining with 5 nM siRNA concentration compared here to 10%. Interestingly, we found out that this disparity resulted from the differently formulated PBNs. The knock-down efficiency of siRNA-loaded WRAP PBNs appeared more efficient when PBNs were first formulated at high concentration and then afterwards diluted, compared to those formulated directly at the desired concentrations (Additional file 3). At this moment, we do not have any rational explanation for understanding this phenomenon and we are performing studies to understand this factual result.

However, because all compared PBNs were formulated and diluted in the same way, the silencing efficiency could be directly compared. W1-2L:siRNA showed a reasonable luciferase knock-down activity even if the effect is not so pronounced compared to W1:siRNA. In contrast, none of the three W5 DL-analogues induced any luciferase silencing (Figure 4A).

All W1-6R, W5-4R and W5-6R siRNA-loaded PBNs showed a dose-dependent luciferase silencing, but slightly less important compared to the parental WRAP PBNs (Figure 4B). In contrast, with a bigger PBN size compared to parent peptide, W1-4R:siRNA had no activity at all used siRNA concentrations.

By looking in details all measured characteristics, we found out that the length of the peptide helix formed in the presence of siRNAs could be a favorable indicator for the PBN silencing activity (Table 1). Knowing that a typical α-helix contains 3.6 amino acids per helical turn [40], we simply calculated the amount of helix turns in the helix depending on the number of amino acids implicated in the helix formation (highlighted in red in the primary sequence in Table 1). Interestingly these numbers directly correlated with the level of the luciferase silencing. No luciferase silencing activities were measured for
peptides forming less than two helical turns (W1-4R and W5-2L). However, if more than two helical turns were present in the peptide, we observed an important luciferase silencing activity suggesting that this minimal helix length was crucial for stable PBN formation and efficient cellular translocation.

However, the overall length of the helical structure in the PBNs could not fully highlight a clear correlation with an optimal silencing efficacy. For example, W1-6R:siRNA with nearly four helical turns showed less silencing activity than the parental W1 peptide (79% vs 91% of knock-down, respectively). A similar effect was observed for W5-6R:siRNA, with a number of helical turns (3.1) comparable to those of the parental peptide W5. In this latter case, the silencing activity dropped down from 88% for W5 to 57% for W5-6R, respectively. To confirm that arginine residue addition had a negative impact on PBN activity, we performed heparin competition experiments to check the stability of the different PBNs. Heparin are sulfated polysaccharide molecules, highly present on the extracellular matrix of cells, which could be able to interact with positive charges of peptides contributing thereby to the siRNA decaging. The heparin sensitivity of PBNs provoking their instability (Additional file 4) could explain the lack of silencing activity. Compared to the parental peptides W1 and W5, only W5-2L and W1-4R showed a higher level of destabilization in good correlation with the lack of silencing activity (Table 1). Whether this lower transfection efficacy was the result of a lower decaging of the siRNA at the cell membrane level or within the cell remains to be fully assessed.

**Evaluating the role of the helix formation in the WRAP sequences.**

To evaluate the importance of the helical structure, and more particularly of its length within the WRAP sequences on PBN formation, we synthesized a new peptide set by integrating one or two proline residues in the middle of their sequences. Indeed, proline residues are unable to form hydrogen bonds within an alpha-helix structure because of the lack of hydrogen on their amide nitrogen. Therefore, proline residues are well-known perturbators of helical structures [40].

As expected, both proline-containing peptides (W1-1P and W5-2P) showed nearly no structural features, whether the analysis was performed using molecular 3D structure prediction (PEPstrMOD) or using circular dichroism analyses (Figures 5A, additional file 1 and additional file 2). In details, CD analyses did not show any major structural changes whether analyses were performed on the peptides alone or associated with siRNAs (Additional file 1-F and additional file 2-G). Once mixed with siRNAs, both peptides W1-1P and W5-2P still formed PBNs, but with diameters of 151 ± 28 nm and 242 ± 60 nm, respectively, slightly bigger than those of the PBNs formed with the corresponding parental peptides (80-100 nm range, see Table 1). When evaluating their luciferase knock-down activity, W1-1P and W5-2P were shown to be 6 to 9 times less efficient, respectively, compared to the parental peptides (Figure 5B). Whether the slight increase in PBN size was responsible for this significant reduction luciferase silencing induced by the siRNA-loaded PBNs remains difficult to be clearly distinguished.
Because the central “RLLRSL” motif of the CADY peptide sequence initiated the helical structure in all hydrophobic media [28], we evaluated if the central leucin doublet could have also an impact on the W1 helix formation by synthetizing a W1 analogue having only one leucin residue in the middle of the sequence (W1-Lm). This D-L peptide analogue showed nearly no structural features as shown by CD measurements (Additional file 1-C) even if the PEPstrMOD prediction revealed a helix of 2.2 turns (Figure 5A). As already exposed, in case of discrepancies between the measured and the expected results about the helical content within peptides, it is worth noticing that we could be more confident in experimental results obtained from CD measurements than in those theoretically predicted. Nevertheless, the formed W1-Lm:siRNA complexes with a mean size of 127 ± 7 nm could induce a luciferase activity silencing of 68% revealing that the deletion of the internal leucin doublet could impact the size (higher) and the activity (lower) of the PBNs.

Finally, we also synthesized W1-mix and W5-mix analogues in which the tryptophan residues were displaced regarding their location in the parental peptides in order to evaluate their influence on the helix formation. Even, if the helicity of the peptides in the presence of siRNA was fully maintained (Additional file 1-G and additional file 2-H), we observed a slightly higher mean size diameter for these PBNs compared to the parent peptides. Concerning the biological effects of these W-mixed peptides, we were surprised to observe differences: W1-mix:siRNA revealed a luciferase knock-down activity of 80% compared to W5-mix:siRNA having only 63% (Figure 5B, Table 1). These results implicated that 4 tryptophan residues could be dispatched along the peptide sequence but that 3 tryptophan residues should be preferably grouped to obtain a comparable luciferase silencing property.

**WRAP nanoparticles compared to lipid-based reagents for siRNA transfection**

Finally, we compared our WRAP-PBNs with other commercially available reagents for siRNA transfection such as RNAiMAX (Life Technologies), INTERFERin (PolyPlus), DharmaFect (Dharmacon) and HiPerFect (Qiagen) selected from their publication score in PubMed.

First, we performed luciferase knock-down experiments strictly under the same conditions. We evaluated silencing properties of the WRAP-PBNs compared to the lipid-based reactants using the classic incubation conditions without removing the transfection solution (1.5 h in serum free-medium + addition of serum-containing medium for 36 h). Unfortunately, compared to the WRAP-based conditions, we observed an important cell mortality (>80%) for most of the lipid-based conditions making the comparison impossible (Data not shown).

Therefore, to avoid cell toxicity, we changed slightly the incubation protocol by removing the whole transfection solution after 1.5 h and adding fresh serum-containing medium for 36 h (Figure 6A). Using these conditions, cytotoxic effects of the lipid-based transfection reagents were reduced under 20% as measured by LDH assay. More importantly, we showed that all transfection reagents (peptide- or lipid-based) were identically active with a specific luciferase silencing of about >80% using a siRNA concentration of 20 nM (Data not shown) or 50 nM siRNAs (Figure 6A). Again, no silencing was recorded with the scrambled siRNA version.
To evaluate the potential long-term effect (over 14 days) of the peptide- or lipid-based transfection reagents, we performed clonogenic assays. This cell survival assay evaluated all modalities of cell death based over a period of two weeks on the ability of a single cell to grow as a colony. In analogy to the luciferase screening, U87 cells were incubated with the different transfection reagents using the same protocol (Figure 6A). We determined a viability threshold corresponding to 100% ± 20% (dotted line in Figure 6B). Under this condition, siRNA alone, W1 and W5 alone or complexed with siRNAs (siLuc or siSCR) have no impact on cell viability with a cell proliferation equal to the non-treated cells.

In contrast, we could highlight important cytotoxic effects for RNAiMAX and DharmaFect, alone or complexed with siRNA (viability ≤20%). For INTERFERin, no cytotoxicity for the transfection reagent alone was recorded, but once complexed to siRNAs, only 30% of cell colonies survived. The only lipid-based transfection reagent without any deleterious effect on cell division was HiPerFect, whether used alone or complexed to siRNAs.

In conclusion, we evaluated 6 different peptide- or lipid-based transfection reagents for their luciferase silencing and long-term cell viability. Only W1, W5 and HiPerfect revealed the same silencing properties (>80% using 50 nM siRNA) without any long-term cytotoxicity compared to RNAiMAX, INTERFERin or DharmaFect. Therefore, W1 and W5 could be used as alternative transfection reagents for inducing an efficient siRNAs internalization and protein knock-down. Once the siRNA delivered into cells, peptides are expected to be fully degraded by proteases and recycled potentially by the cells.

Discussion

By performing a structure-activity relationship (SAR) study with our lead WRAP1 (W1) and WRAP5 (W5) peptides [16,30] together with 13 newly synthesized analogues, we have evaluated the role of the leucine, arginine and tryptophan residues with the WRAP peptide sequences as well as the impact of the structural peptide changes after the addition of siRNAs.

In the first step of our SAR study, we showed the higher luciferase inhibition rate of the WRAP:siRNA PBNs compared to the C6M1 and C6M1-L peptides. Because of the close similarity in amino acid content between these two families of peptides, we wanted to understand why these peptides behaved differently in their ability to transfect siRNAs into cells. According to the publication of our colleagues[37], measurement by Dynamic Light Scattering (DLS) and microscopy techniques revealed a particle size with a diameter of 70 nm for the C6M1-siRNA in water. This diameter was closed to the diameter observed for the WRAP:siRNA PBNs (around 80 nm as shown in [16] and herein Table 1). Therefore, we cannot consider the particle size as the main criterion to explain this discrepancy in the transfection rate between these peptides. But for all our analogues evaluated in this work, it was noteworthy that PBNs with a diameter higher than about 250 nm did not induce any biological effects in our siRNA-Luciferase assay.
The clearer difference between the two series of peptides relies on the number of arginine, higher in the C6M1 series (7 residues) compared to the WRAP series (4 residues). Arginine residues were expected to interact directly with the phosphate groups of siRNAs through ionic interactions during the PBNs formation. They were also responsible of the translocating process since peptides made exclusively of arginine residues (poly-Arg) were able to translocate efficiently into cells [32]. Logically, we could hypothesize the C6M1 to be more efficient regarding this aspect, but we faced exactly the opposite (Figure 1B). One possibility remains the slower or less efficient decaging of the siRNA once the PBNs reaches the cytosol for a peptide made with more arginine residues. This was highlighted in our experiment when heparin was used to displace the binding of siRNAs from the different peptides. The higher number of arginine residues in peptides required a higher concentration of heparin to destabilize the PBNs.

In line with this, Jafari and colleagues [23] also demonstrated that the molar ratio of C6M1 peptide required to completely encapsulate siRNAs was in the same range than the molar ratio used with our WRAP peptides (both requiring an optimal ratio of peptide over the siRNA of R= 15 as observed in a gel-shift retardation assay in our respective laboratories). In our comparative experiment, we used the same ratio for both peptide series. Consequently, the charge ratio between C6M1 and WRAP peptides over the siRNAs can be calculated as being 3.5 cations/anion for C6M1 and 2.2 cations/anion for WRAP because of the presence of 7 arginine residues in C6M1 versus 5 in the WRAP peptides. There was thus an excess of arginine residues over anionic groups from the siRNA in the C6M1 complexes thus possibly impairing an efficient cellular decaging of the siRNAs from the PBNs. We cannot exclude this difference in the lower inhibitory response of C6M1 to be the direct consequence of its higher arginine content.

Moreover, we also observed that the deletion of one single leucine residue from a leucine doublet within the C6M1 peptide (C6M1-L) could reduce of about 50% the luciferase silencing mediated by the parental peptide. Therefore, we also wanted to verify whether the leucine doublets, mainly present in our WRAP peptides at both extremities, but also in the middle of the sequence, were important in the PBN formation as well for the siRNA-induced luciferase silencing. Despite the ability for all these D-Leu peptides to bind siRNAs with roughly the same level in a gel-shift assay (Figure 2A), their respective ability to induce a biological silencing effect was rather different (Figure 4A). W1 peptide deleted from the doublets at the extremities (W1-2L) was still active, while the deletion of leucine residues doublets at any position in W5 was highly deleterious (Figure 4A). In these latter cases however, the size of the formed nanoparticles with the W5 peptide series led to aggregates with a diameter higher than 1,000 nm, while the PBNs made with the W1 peptide deleted from its leucine (W1-2L and W1-Lm) remained in the 100 nm range. Despite the ability of these peptides to also induce the expected biological response, we noticed their instability upon 4 days storage at 4°C. Leucine doublets thus appeared to positively influence the formation of PBNs with a size acceptable for promoting the intracellular transport of siRNAs.

We also related the efficacy of our WRAP peptides and analogues with the length of the helical structure formed once the peptides were combined with the siRNAs. The length of the helix was also directly correlated with the size of the resulting PBNs showing that peptides structured in short helix (below 2
helical turns) formed bigger PBNs. The single exception was the peptide W1-1P (with a proline residue in the middle of the sequence) having a short helix (only 1.4 helical turn), forming 150 nm sized PBNs and showing a weak ability to extinguish the targeted luciferase expression in our transfection assay (Figure 5B). However, compared to the other peptides, that one was strongly unstable upon a 4 day-period, while parental W1 and W5 peptides were stable for weeks or months.

In our previous work [16], we showed that the number of tryptophan residue was important to promote PBN formation and subsequently, the extinction of the targeted protein expression. Herein, we investigated whether their location within the sequence could influence the overall efficacy of the transfection. With the exception of W5-mix (not stable and showing low silencing), we did not observe a spectacular effect associated with the relative tryptophan position within the WRAP sequences.

Based on the results described in this work and those obtained previously [16], we established for WRAP peptides and WRAP-PBNs the following consensus motif \( \text{LL-[X]}_n\text{-LL} \) with \( n = 11 - 12 \) amino acids including the three restrictions for X:

- The number of internal arginine residues should be \( n = 4 \)
- The number of internal leucine residues should be \( n = 4 \) and they required to be inserted as doublet.
- The number of internal tryptophan residues should be \( n = 3 \) for a grouped tryptophan localization in the middle of peptide sequence (WWW) or \( n = 4 \) for a grouped (2x WW) or a clustered through secondary structure folding (4x single W).

Further rules concerning the amino acid composition are difficult to postulated. To obtain stable PBNs with a maximal silencing activity (~80% or more), we recommend to use PBNs with a mean size between 80 nm and 110 nm as well as a minimal helix turn of 2.5 because the helix length seems to positively influence the PBN mean size (e.g. W1-4R: 1.9 helix turn, 441 nm \textit{versus} W5-6R: 3.1 helix turn, 64 nm). However, the helix length did not automatically influence the PBN stability as shown for W1-2L which is not stable after a 4 day-storage at 4°C.

Finally, two approvals of siRNA therapeutics, ONPATTRO® (patisiran) and GIVLAARI™ (givosiran), have been recently achieved for the treatment of hereditary amyloidogenic transthyretin (hATTR) amyloidosis in adults and for the treatment of acute hepatic porphyria (AHP), respectively. Several other therapeutics siRNAs are in the latest phases before claiming for market agreements. In most of the cases, delivery systems for siRNAs are based on materials derived from lipids, lipid-like materials, polymers, exosomes or inorganic nanoparticles. Here we showed that peptides W1 and W5 appeared as good alternative transfection agents to promote efficiently siRNA cellular internalization compared to commonly used transfection reagents made of cationic lipids. If the overall efficacy of the WRAP peptides and the non-peptide transfection reagents could be comparable, we noticed an important difference in the cell viability in a long-term clonogenic assay. Only one out of the four reagents we tested showed none toxicity as recorded for the WRAP peptides. Therefore, these WRAP peptides could be used as efficient and safe
compounds to extinguish the expression of any kind of endogenous or exogeneous proteins with a wide number of cell-types as recently demonstrated by our group [16].

Materials And Methods

Materials: WRAP peptides were synthesized on the SynBio3 platform (IBMM Montpellier) and crude products were purified in house following a qualitative analysis by HPLC/MS. siRNA (siFLuc: 5'-CUU-ACG-CUG-AGU-ACU-UCG-AdTdT-3' as sense strand and siSCR: 5'-GAA-UGC-GAC-UCA-UGA-AGC-UdTdT-3') were purchased from Eurogentec. The following siRNA transfection reagents were used: RNAiMax (Life Technologies), INTERFERin (PolyPlus), DharmaFECT (Dharmacon) and HiPerFect (Qiagen).

Nanoparticle formation: Stock solutions of WRAP peptides (see Table 1) and of siRNA were prepared in pure water and in RNase-free water, respectively. Nanoparticles were formulated in pure water supplemented by 5% (m/v) glucose by adding first the CPP and then the corresponding amount of siRNA at molar ratio (R) of 20 (WRAP:siRNA = 20:1) at room temperature. Formulated PBNs could be stored for several weeks at 4°C without loss of transfection efficacy.

Peptide structure prediction: PEPstrMOD server was used to predict the secondary structure of WRAPs (http://osddlinux.osdd.net/raghava/pepstrmod/) [38,39].

Circular dichroism (CD) measurements: CD spectra were recorded on a Jasco 810 (Japan) dichrograph in quartz suprasil cells (Hellma) with an optical path of 1 mm for peptide in solution or in the presence of liposomes vesicles. Same peptide concentrations (40 µM) were used for each condition. Spectra were obtained from 3 accumulations between 190 nm and 260 nm with a data pitch of 0.5 nm, a bandwidth of 1 nm and a standard sensitivity.

Agarose gel-shift retardation assay: WRAP:siRNA complexes [siRNA = 10 µM in 20 µL of an aqueous solution containing 5% glucose] were formed at different ratios and pre-incubated for 30 min at room temperature. Then 20 µL were loaded on an agarose gel (1 % w/v, Sigma-Aldrich) and electrophoresis was performed at 50 V for 25 min. To visualize the siRNA the agarose gel was stained with GelRed (Interchim) for UV detection and images were acquired using the Amersham 600 imager (GE Lifesciences). The signal intensities were quantified after background subtraction using Image J software (gel analyze tool). Each band intensity corresponding to a distinguished condition was then normalized to the band intensity of the siRNA alone (= 100%): Relative fluorescence (%) = fluorescence intensity (condition x)/fluorescence intensity (siRNA alone) x 100.

Dynamic light scattering (DLS): WRAP:siRNA nanoparticles (WRAP = 10 µM, siRNA = 500 nM, R = 20) were evaluated with a Zetasizer NanoZS (Malvern) in terms of mean size (Z-average) of the particle distribution and of homogeneity (PDI). All results were obtained from three independent measurements (three runs for each measurement at 25°C).
Cell culture conditions: Human glioblastoma cell line (U87) overexpressing the firefly and nanoluc luciferase (FLuc-NLuc) were kindly provided by Dr. Franck Couillaud (Bordeaux, France). Cells were grown in DMEM-pyruvate-GlutaMAX™ medium (Life Technologies), supplemented with 1% penicillin/streptomycin (Life Technologies), 10% heat-inactivated fetal bovine serum (FBS, ThermoFisher), 0.1% non-essential amino acids (NEAA 1X, LifeTechnologies) and 0.01% hygromycin B (50 µg/mL, Invitrogen). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Human macrophage cell line (U937) overexpressing the firefly luciferase was kindly provided by Dr. Guillaume Bossis (Montpellier, France). Cells were grown in RPMI medium (Life Technologies), supplemented with 1% penicillin/streptomycin (Life Technologies), 10% heat-inactivated fetal bovine serum (FBS, ThermoFisher). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C.

Luciferase assay. U87 cells (5,000 cells/well) were seeded 24 h before experiment into 96-well plates. Before PBN incubation, the cell growth medium was replaced by 70 µL of fresh pre-warmed serum-free DMEM. Afterwards, 30 µL of the PBN solutions were added directly to the medium recovering the cells and incubated 1.5 h at 37°C. Finally, 100 µL fresh DMEM supplemented with 20% FBS were added to the cells (10% FBS at final concentration) and the cells were incubated for further 36 h. The medium covering the cells was then carefully removed and 50 µL of 0.5 X Passive Lysis Buffer (PLB; Promega) were added for 30 min cell lysis at 4°C. After a centrifugation step (10 min, 1,800 rpm, 4°C), 10 µL of each cell lysate supernatant were transferred into a white 96-well plate (Greiner Lumitrac™ 200). Luciferase activity was quantified using a plate-reading luminometer (POLARstar Omega, BMG Labtech) and half-diluted dual luciferase assay reagents as described by the manufacturer (Promega). The results were expressed as percentage of relative light units (RLU) for each luciferase, normalized first to non-treated cells (%FLuc and %NLuc) and then to the value of %NLuc to obtain the relative Luc activity (%FLuc/%NLuc).

Cytotoxicity assay: The Cytotoxicity Detection KitPlus (LDH, Roche Diagnostics) was used to evaluate the cytotoxicity induced by the PBNs. After the PBN incubation (36 h), at least one well was used as a LDH positive control (100% toxicity) by adding Triton X-100 (Sigma-Aldrich) to a final concentration of 0.1% (~15 min incubation at 37°C). Afterwards, 50 µL supernatant of each well were transferred in a new clear 96-well plate (Greiner) and completed with 50 µL/well of the “dye solution/catalyst” mixture as recommended by the manufacturer. The plate was then incubated in the darkness for 30 min at room temperature. Reaction was stopped by adding 25 µL/well of HCl (1 N) before measuring the absorption at 490 nm using the POLARstar Omega plate reader (BMG Labtech). Relative toxicity (%) was calculated with the following formula: [(exp. value – value non-treated cells) / (value triton – value non-treated cells)] x 100.

Clonogenic assay: U87 cells (450 cells per well) were seeded 24 h before experiment into 6-well plates. Before PBN or lipid-based transfection reagent incubation, the cells were washed twice with D-PBS (Life Technologies). 700 µL of fresh pre-warmed serum-free DMEM and 300 µL transfection solutions were added to the cells and incubated 1.5 h at 37°C. Afterwards, solutions recovering the cells were supplemented by 2 mL fresh DMEM supplemented with 10% FBS and the cells were incubated for further
10 days at 37°C. At the end of the incubation, cells were first fixed using a solution of methanol/acetic acid (3:1) at 4°C for 20 min. The fixed cells were colored using a freshly prepared Giemsa solution (Sigma-Aldrich) diluted in water (3.5:10) for 20 min at room temperature. To visualize the colonies, each well was abundantly washed with water and then dried. Images of the colonies were acquired using the Amersham 600 imager (GE Lifesciences). Colonies for each well were counted and normalized to the non-treated cells.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Author contributions Statement**

E.V., K.K., S.D. and P.B. conceived the design of the study; M.T., K.R., E.J., and K.K. performed research; K.K., S.D., G.A., E.V. and P.B. analyzed data. P.B. and E.V. wrote the paper; E.J., G.A., S.D. and K.K. revised the manuscript.

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