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The agony of choice: how to find a suitable CPP for cargo delivery

Judith Müller,[a] Julia Triebus,[a] Ines Kretzschmar,[a] Rudolf Volkmer[a] and Prisca Boisguerin[a,b]*

Successful and effective cellular delivery remains a main obstacles in the medical field. The use of cell-penetrating peptides (CPPs) has become one of the most important tools for the internalisation of a wide range of molecules including pharmaceuticals. It is still difficult to choose one CPP for one biological application because there is no ubiquitous CPP meeting the diverse requirements. In our case, we are looking for a suitable CPP to deliver the pro-apoptotic KLA peptide (KLAKLAKKLAKLAK) by a simple co-incubation strategy. For that reason, we selected three different cell lines (fibroblastic, cancerous and macrophagic cells) and studied the uptake and subcellular localisation of six different CPPs alone as well as mixed with the KLA peptide. Furthermore, we used the CPPs with a carboxyamidated or a carboxylated C-terminus and analysed the impact of the C-termini on internalisation and cargo delivery. We could clearly showed that the cellular CPP uptake is not only dependent on the used CPP and cell line but also highly affected by its chemical nature of the C-terminus (uptake: carboxyamidated CPPs > carboxylated CPPs) and can influence its cellular localisation. We successfully delivered the KLA peptide in the three cell lines and learned that here as well, the C-terminus is crucial for an effective peptide delivery. Finally, we induced apoptosis in mouse leukaemic monocyte macrophage (RAW 264.7) and in human breast adenocarcinoma (MCF-7) cells using the mixture of amidated MPG peptide : KLA and in african green monkey kidney fibroblast (Cos-7) cells using carboxylated integrin peptide : KLA.

Supporting information may be found in the online version of this article.

Keywords: cell-penetrating peptide; KLA peptide; pro-apoptotic; non-covalent; peptide delivery

Introduction

Intracellular delivery of therapeutic molecules is one of the major road blocks in drug transportation having their curative action in the cytoplasm or individual organelles such as nuclei or mitochondria. Since the first report of a cell-penetrating peptide (CPP) in 1994, a stream of CPPs displaying membrane-translocation properties has emerged to overcome drug delivery problems [1]. To date, no distinct definition of the CPP family could be determined; only common features have been agreed on, such as (i) CPPs carry a net positive charge and (ii) CPPs facilitate the rapid translocation of molecules across cellular membranes. They consist of less than 30 amino acids and are mostly amphiphathic. The mechanism by which CPPs internalise into cells has been the focus of many research articles and is thought to involve direct translocation over the membrane via micelle and pore formation [2] but is considered mostly to occur by different types of endocytosis [3,4].

Cell-penetrating peptides show remarkable cell-penetrating properties in various cell types (cell lines, primary cultures or tissues) and have transported a range of different molecules, e.g. nucleic acids, PNA, peptides, proteins, liposomes and nanoparticles [5,6].

However, despite the high number of biological applications using CPPs, there is no distinct rule or general protocol for the use of them. This means that information can only be gathered piece by piece from different sources, and it is difficult to select one specific CPP for one distinct application. For that reason, a few years back, we analytically screened 22 CPPs in four cell lines [Madin-Darby canine kidney (MDCK), human embryonic kidney (HEK293), human cervix carcinoma (HeLa) and Cos-7] and measured the cellular uptake using a microplate reader (cell lysis) or confocal microscopy [7]. Altogether, we clearly demonstrated the necessity for detailed analysis of the used CPP, the used cell line and the applied working conditions before the implementation in a biological context.

Having now a subset of CPPs in our hand, which have different cell-penetrating properties, our new orientation lies in the analysis of the delivery of a biologically active peptide, e.g. a therapeutic peptide. Therapeutic peptides are a promising new sort of drug because of their potential great target specificity, easy development and low-prized production [8]. One class of therapeutic peptides are the pro-apoptotic peptides that hold a lot of perspective in the treatment of cancer. A widely used pro-apoptotic peptide is the polycationic KLA peptide (KLAKLAKKLAKLAK) [9,10]. The pro-apoptotic KLA peptides contain highly positively charged sequences, which direct the internalised peptide to the mitochondrial membrane but have a low affinity for the plasma membrane and are extracellular non-cytotoxic to mammalian cells [10].

One of our main goals was to internalise the pro-apoptotic KLA peptide via a non-covalent strategy achieved using a simple

* *Correspondence to: Prisca Boisguerin, UMR 5235 CNRS, Universités Montpellier 1 et 2, Place Eugene Bataillon, 34095 Montpellier, France. E-mail: prisca.boisguerin@univ-montp2.fr*

a *Institute of Medical Immunology, Charité, Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany*

b *UMR 5235 CNRS, Universités Montpellier 1 et 2, Place Eugene Bataillon, 34095 Montpellier, France*

co-incubation procedure of both – CPP and KLA peptides. Besides finding the optimal CPP for our application, we evaluated the influence of carboxylated and carboxyamidated CPP C-termini on the peptide's uptake as well as on the delivery of the KLA peptide because Simeoni *et al*. stated that the structure of the C-terminus is important for cellular transduction [11].

For our study, we selected six CPPs that were then analysed in MCF-7, RAW 264.7 and Cos-7 cells in terms of cellular uptake, cellular localisation and efficacy of KLA internalisation dependent on their C-termini. After the determination of the optimal mixing ratio (CPP : KLA), we identified the most efficient mixture as MPG-NH_2 : KLA (1 : 2) for MCF-7 and for RAW 264.7 cells, revealing the highest pro-apoptotic KLA effect. Because the MPG-NH_2 : KLA mixture has no effect on Cos-7 cells, this condition holds potential for the therapeutic treatment of cancer.

Results

Careful CPP selection for a specific cell system is important [7]. On the basis of our previous results, we selected six CPPs with low, medium and high uptake (penetratin, Tat and integrin peptides, $S4_{13}PV$, MPG and R9; Table 1) that were synthesised with a carboxyamidated (-NH_2) or carboxylated (-COOH) C-terminus to analyse its influence on the cellular internalisation, its intracellular distribution as well as its influence on the delivery of the KLA peptide. In terms of a therapeutical application, all *in vitro* experiments were performed in MCF-7 cells (adenocarcinoma cells) and in RAW 264.7 cells (leukaemic macrophages) in comparison to the Cos-7 cells (fibroblasts).

First of all, we determined potential cytotoxic effects of the CPPs (-NH_2 vs -COOH) using the MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) viability assay. Fortunately, most of the CPPs did not influence the cellular viability even at a high concentration of 50 μM and 2-h incubation independently of their C-terminus. Only $S4_{13}$PV-NH_2 revealed toxic properties (e.g. 14% ± 11% viability in MCF-7 and 37% ± 3% in RAW 264.7 cells at 10 μM) (Supporting Information Table S1).

The three cell lines (Cos-7, MCF-7 and RAW 264.7) were incubated with the CF-labelled CPPs listed in Table 1 and their internalisation was quantified by fluorescence measurements using a microplate reader. The uptake measurements shown in Figure 1A clearly revealed a strong influence of the CPP chemical nature of the C-terminus on CPP cellular uptake (values are given in Supporting Information Table S2). The internalisation of the carboxyamidated form of the penetratin peptide, R9 and integrin peptide is extremely improved in all cell lines compared to the carboxylated form. In contrast, the uptake of the Tat-peptide and $S4_{13}PV$ is nearly not influenced when changing the C-terminus in all analysed cell lines. Curiously, the C-terminus of MPG seems to have no influence on the uptake in MCF-7 cells, which corresponds in both cases to one of the highest measured values (>12,000 CF-SI/mg protein).

Table 1. Sequences of the used peptides

CPP	Sequences	Reference
Penetratin peptide	RQILIWFQNRRMKWKK	[1]
Tat peptide	GRKKRRQRRRPPQ	[25]
Integrin peptide	VTVLALGALAGVGVG	[26]
$S4_{13}PV$	ALWKTLLKKVLKAPKKKRKV	[27]
MPG	GALFLGWLGAAGSTMGAWSQPKKKRKV	[28]
R9	RRRRRRRRR	[29]
KLA	KLAKLAKKLAKLAK	[9]

Cell-penetrating peptides were synthesised as amide (−NH_2) or as carboxylate (−COOH). Furthermore, CPPs were N-terminally labelled with (5/6) carboxyfluorescein (CF-) and carboxyamidated KLA with Cy5 for uptake and microscopic analyses.

Differences in the amount of the cellular uptake between carboxylated and carboxyamidated CPPs could also result in different subcellular localisations, which were analysed using confocal microscopy (Figure 1B and Supporting Information Figure S1). First, no auto-fluorescence was detected in all cell lines, and cell integrity was shown by Trypan blue staining. Using the same conditions as in the microtiter plate reader experiments, we were able to confirm our results and detected a stronger CF–fluorescence signal in the corresponding samples. For most cell lines, it was not possible to identify a change in the subcellular localisation of the CPPs when altering the C-termini – they showed a punctuated pattern throughout the cytoplasm, which probably resulted from uptake via endocytosis [3]. Interestingly, only MCF-7 cells exhibited a different distribution for carboxylated and carboxyamidated peptides. The carboxyamidated penetratin peptide, MPG and $S4_{13}PV$ were localised not only in the endosomes as seen for their carboxylated forms but also diffused in the cytoplasm as well as in the nucleus.

In summary, the structure of the C-terminus has an influence on (i) the cellular CPP uptake (carboxyamidated CPPs > carboxylated CPPs) and in a few cases on (ii) the CPP's cellular localisation depending on the CPP and cell line.

Optimal Mixing Ratio for the Non-Covalent Delivery of the KLA Peptide

A therapy using biological active peptides (e.g. for cancer treatment) should be flexible in the application. For that reason, we decided to apply the pro-apoptotic peptide KLA [9] by simply mixing it with the CPPs (non-covalent strategy). To ensure the best cellular delivery of the KLA peptide, we tried to determine the optimal mixing ratio using MPG-NH_2 : KLA and Int-NH_2 : KLA in Cos-7 cells (both CPPs show high internalisations in Figure 1A). We co-incubated the cells with Cy5-labelled KLA and CF-labelled CPP-NH_2 in different molar ratios (20 : 1 to 1 : 2 for CPP : KLA) as well as with KLA and CPP alone (Figure 2A). Interestingly, the internalisation was dramatically reduced using ratios of 10 : 1 or 20 : 1 (CPP : KLA) that are typical ratios for CPP : oligonucleotide mixtures [12]. Furthermore, we incubated Cos-7 cells with MPG-NH_2 : KLA mixtures that were pre-incubated for 30 min before adding them to the cells (Figure 2A). Because there was no significant difference to the incubation without a pre-formulation step, we decided to skip the pre-incubation step.

In detail, we observed an increased cellular uptake of the KLA peptide when mixed to the appropriate same amount of CPP compared with the incubation without one. For example, incubating Cos-7 cells with KLA together with MPG-NH_2 or Int-NH_2 roughly doubled or tripled the amount of uptake [e.g. 390 ± 95 Cy5-SI/mg protein for KLA alone vs 1,106 ± 230 Cy5-SI/mg protein for Int-NH_2 : KLA (1 : 2)]. We also checked whether a CPP with low cellular uptake such the Tat peptide was able to translocate the KLA peptide using the non-covalent strategy. The results shown in Figure 2A clearly demonstrate

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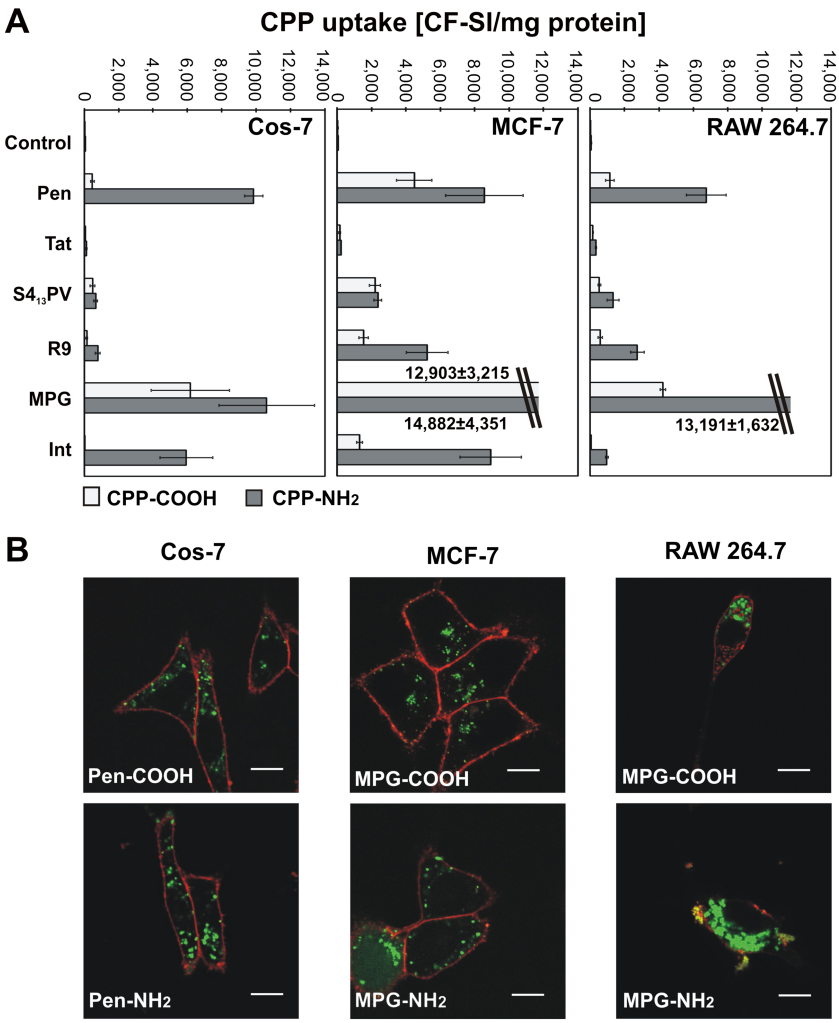

Figure 1. Role of the C-terminus on CPP internalisation: (A) Cellular uptake: Cos-7, MCF-7 and RAW 264.7 cells were incubated for 30 min with six selected CPPs having either a carboxylate or carboxyamidated C-terminus. Afterwards, cells were washed, trypsinised, lysed and analysed using a microtiter reader. Data shown are the means ± SEM ($n = 3$). (B) Cellular distribution of three CPPs: Cos-7, MCF-7 and RAW 264.7 cells were incubated with 10 μM CPPs for 30 min, washed and analysed. For each condition, we show the overlay of the CF-CPP staining (488 nm) and the Trypan blue staining (543 nm). The white bars represent 10 μm. Carboxyamidated and carboxylated CPPs were differently localised only in MCF-7 cells. The CPP's C-terminus did not influence the subcellular localisation in the other cell lines.

that co-incubating the Tat peptide with KLA did not improve the internalisation, confirming the importance of CPP selection for cargo delivery.

The same mixing ratios resulted in high uptake amounts as well, when we used a different arbitrary chosen peptide (PRC, sequence ANSRWQTSII, Figure 2B). Therefore, we believe that this phenomenon was not dependent on the KLA sequence as cargo (positively charged, helix conformation) (Figure 2B) [9]. Even though the overall uptake amounts were much smaller for PRC than for KLA, its internalisation was doubled or tripled when mixed with MPG-NH_2 or Int-NH_2 at ratios of 1 : 1 or 1 : 2 compared with the internalisation without a CPP as transport vehicle [104 ± 26 Cy5-SI/mg protein for PRC alone vs 310 ± 69 Cy5-SI/mg protein for MPG-NH_2 : PRC (1 : 2)]. Here as well, the internalisation was rather dependent on the used CPP as well as on its chemical nature of the C-terminus.

Our results suggest that mixing ratios of 1 : 1 and 1 : 2 (CPP : cargo peptide) are the best choices for the delivery of a peptide mixed with a CPP. For all following experiments, we chose these ratios to evaluate the uptake and induce pro-apoptotic effects with the transduced KLA peptide.

Cellular Delivery of the KLA Peptide

Having found the optimal mixing ratios (1 : 1 or 1 : 2), we started to incubate the three cell lines with KLA mixed with MPG, integrin and penetratin peptides and tested them in both versions (carboxylated vs carboxyamidated) (Figure 3 and Table S3).

The KLA delivery in Cos-7 cells is mainly possible with penetratin and integrin peptides. However, the internalisation depends on the C-terminus of the CPP, especially in the case of the integrin peptide. We observed a fivefold to sixfold increase in KLA uptake with Int-COOH compared with Int-NH_2 at both ratios (5,085 ± 803 Cy5-SI/mg protein vs 987 ± 197 Cy5-SI/mg protein for ratio 1 : 1 and 5,737 ± 705 Cy5-SI/mg protein vs 1,106 ± 230 Cy5-SI/mg protein for ratio 1 : 2).

Applying the same conditions of KLA delivery (10 μM CPP with 1 : 1 or 1 : 2 ratio) in MCF-7 and RAW 264.7 cells, we observed a rapid detachment of the cells making the analysis of the results difficult. For that reason, we performed these assays using a tenfold diluted concentrate (1 μM CPP with ratio 1 : 1 or 1 : 2). As in Cos-7 cells, we saw an enhancement of the KLA internalisation in both cell lines depending on the CPP C-terminus, even if the

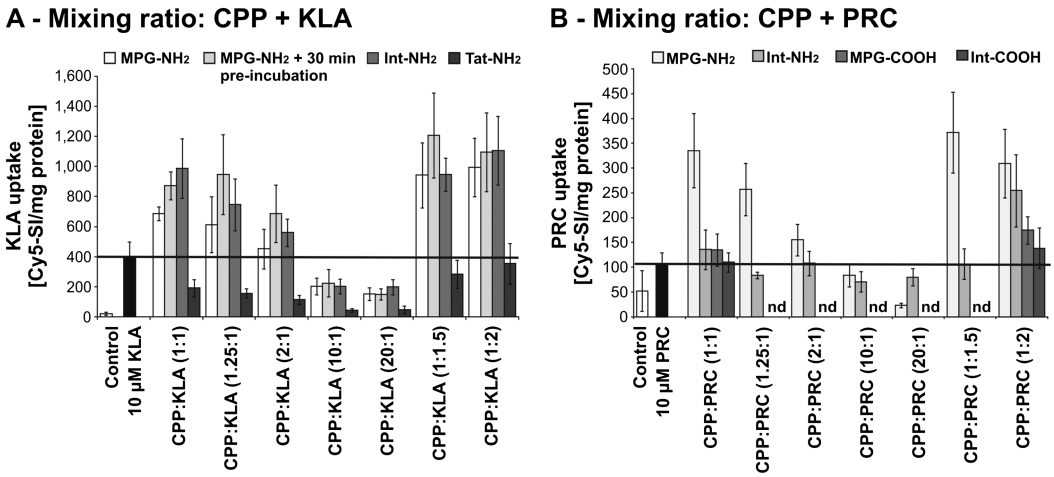

Figure 2. Mixing ratios of the (A) KLA or (B) PRC peptide and CPPs: Cos-7 cells were incubated with 10 μM CPP and different concentrations of the KLA or PRC peptide for 30 min. For all conditions, the peptides were co-incubated; only in (A) MPG-NH_2 : KLA solutions were pre-incubated for 30 min. The Cy5-KLA/PRC uptake was measured and normalised to the total protein concentration (Cy5-SI/mg protein). The CF-CPP uptake was also monitored to ensure consistency (data not shown). The maximal uptake was reached using CPP : KLA mixture ratios of 1 : 1 and 1 : 2. n.d. means 'not determined'. Data shown are the means ± SEM ($n = 3$).

signal was weaker because of lower peptide concentrations. We determined the best results with MPG-COOH : KLA (1 : 2) (423 ± 61 Cy5-SI/mg protein; threefold increase compared with KLA alone) for MCF-7 and MPG-NH_2 : KLA (1 : 2) (734 ± 67 Cy5-SI/mg protein 2.5-fold increase compared with KLA alone) for RAW 264.7 cells.

For each KLA uptake experiment, we always measured the CF-CPP uptake rates and saw no difference in the incubation with just the CPP or with the CPP : KLA mixtures (Supporting Information Figure S2). Therefore, we believe that the KLA peptide has no influence on the CPP uptake as well as on the CF–fluorescence intensities.

Using confocal microscopy, we could confirm the results found using microtiter plate reader measurements: high uptake corresponded to high fluorescence signals in the analysed cells (Figure 3B). For example, the vast difference in transportation capability of both forms of the integrin peptide was confirmed for Cos-7 cells (Int-COOH : KLA >> Int-NH_2 : KLA). The higher delivery by Int-NH_2 at a ratio of 1 : 2 compared with 1 : 1 in MCF-7 cells as well as MPG-NH_2 1 : 2 in relation to 1 : 1 in RAW 264.7 cells were also validated.

Looking at the pictures in greater detail, we discovered a huge accumulation of CF-Int-COOH : Cy5-KLA around the nucleus of Cos-7 cells (merged distribution in orange, Figure 3B). We also saw a strong cellular accumulation for CF-Int-NH_2 : Cy5-KLA (1 : 2) in MCF-7 and for CF-CPP : Cy5-KLA (1 : 2) in RAW 264.7 cells, which correspond to the values measured in the uptake tests – even at a low concentration (1 μM CPP). Interestingly, the cellular distribution seemed to be mainly cytosolic, hinting towards a potential entrapment in endosomal vesicles. Because there was also a diffuse signal detectable surrounding the vesicles, we believe, KLA might be able to escape the entrapment.

Activity of the CPP-Delivered Pro-Apoptotic KLA Peptide

To evaluate the activity of the pro-apoptotic KLA peptide introduced by the MPG, integrin or penetratin peptides, we utilised the MTT test to assess the cell viability using the same conditions as in the KLA delivery experiments. A value of 100% corresponds to a non-toxic effect of the peptides, whereas values lower than 80% viability represent a toxic effect. First of all, we observed that CPPs and the KLA peptide themselves caused no toxicity.

In Cos-7 cells, most combinations of CPPs and KLA did not lower the cell viability significantly (Figure 4A). A detectable pro-apoptotic effect with a cell viability reduction of 32% ($p < 0.001$ vs KLA alone) was only observed with the combination of 10 μM Int-COOH and 20 μM KLA.

In MCF-7 cells, we observed in 58% of the applied conditions a reduction in cell viability although using tenfold lower concentrations than in Cos-7 cells (Figure 4B). The highest effects were given by MPG-COOH : KLA (1 : 2) and MPG-NH_2 : KLA (1 : 2) with reductions of 32% ($p < 0.001$ vs KLA alone) and 42% ($p < 0.001$ vs KLA alone), respectively. This is in agreement with the results of the cellular uptake, where both were amongst the combinations with the highest delivery rates.

In RAW 264.7 cells, the toxic effects of KLA delivered by different CPPs and changing ratios were not as obvious as in MCF-7 cells (Figure 4C). Using the same condition as applied for the MCF-7 cells (MTT detection after 3 days), we did not observe a pro-apoptotic effect for any of the used mixing partners. Therefore, we evaluated the toxicity of the KLA peptide by reducing the period after peptide incubation (Supporting Information, Figure S3). Measuring the cell viability directly after peptide incubation, we determined a viability reduction of 27% ($p < 0.05$ vs KLA alone) with 1 μM MPG-NH_2 + 2 μM KLA; a combination that caused toxicity in MCF-7 cells as well. Toxicity was also observed using 1 μM Pen-NH_2 + 2 μM KLA with a viability reduction of 26% ($p < 0.05$ vs KLA alone).

MCF-7 and macrophagic RAW 264.7 cells showed the highest induction of apoptosis with MPG-NH_2 : KLA (1 : 2), which was not found in fibroblastic Cos-7 cells even at a tenfold higher concentration.

To ascribe the viability reductions measured with the MTT tests to pro-apoptotic effects of the CPP : KLA mixtures, we investigated the nuclear morphology of Cos-7, MCF-7 and RAW 264.7 cells to detect nuclear pyknosis, which is a sign of cells undergoing apoptosis. We used the conditions that showed toxic effects in the MTT assay and stained the cells with Hoechst 33342. Cos-7 cells showed no altered nuclei in the control picture or when incubated with MPG-NH_2 : KLA (1 : 2), but condensed

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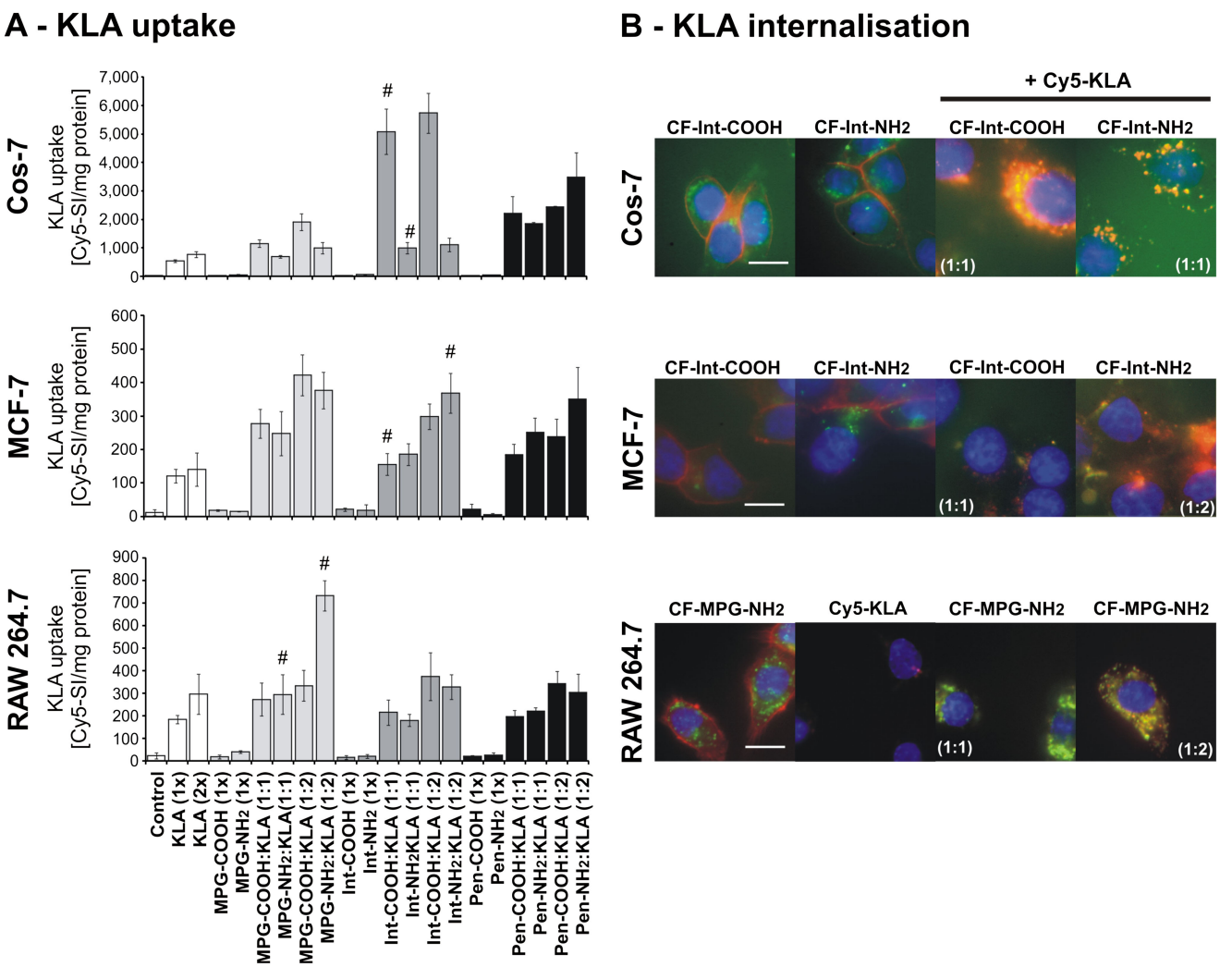

Figure 3. Cellular delivery and distribution of the pro-apoptotic KLA peptide mixed with a CPP: (A) KLA delivery by CPPs in Cos-7, MCF-7 and RAW 264.7 cells: Cells were incubated with CPPs and KLA as described before. '1×' means 1 or 10 μM and '2×' means 2 or 20 μM depending on the cell lines. We were able to successfully enhance the delivery of KLA by mixing it with a CPP in all cell lines. Notice the strong enhancement with Int-COOH in Cos-7 and MPG-NH_2 in RAW 264.7 cells. Data shown are the means ± SEM ($n = 3$). (B) Cellular distribution of the KLA peptide: Combinations of CPP and KLA with the symbol # in Figure 3A were analysed under the microscope. Trypan blue was added to ensure cell viability but omitted when KLA was present to avoid spectral overlaps. Nuclei were stained with Hoechst 33342. CF-CPPs are visualised in green, Trypan blue and Cy5-KLA in red. The scale bars represent 10 μm.

chromatin was found after the incubation with Int-NH_2 : KLA (1 : 2) (white arrows in Figure 4D). In MCF-7 cells, nuclear pyknosis was clearly visible after the incubation with MPG-NH_2 : KLA (1 : 2) after 2 days and even more pronounced after 3 days (Figure 4E). When incubating RAW 264.7 cells with MPG-NH_2 : KLA (1 : 2), degenerated cell nuclei were only detectable at the day of the incubation (Figure 4F) but not after 3 days (data not shown). Furthermore, using the Int-COOH : KLA (1 : 2) condition, no effect was identifiable, which coincides with the MTT assays.

Finally, with regard to a potential pharmaceutical application in cancer treatment, we related the viability reduction in the adenocarcinoma cells MCF-7 to apoptosis by annexin-V staining and DNA fragmentation analysis (Supporting Information Figure S4).

Discussion

Cell-penetrating peptides are proven vehicles for the intracellular delivery of macromolecules such as oligonucleotides, peptides and proteins; low-molecular-mass drugs; nanoparticles; and liposomes [13]. Despite the high number of biological applications using CPPs, a lot of questions remain unanswered such as (i) what is the fundamental mechanism of translocation across biological membranes and (ii) how does the 'cargo' affect the efficiency and mechanism of action of CPPs. However, even if there is no 'golden rule' for the CPP application, the importance of CPPs for controlled and targeted delivery of therapeutic and imaging agents is very clear. Mechanisms of cellular uptake of CPPs are still not completely understood; endocytotic and non-endocytotic routes have been suggested and observed [14,15]. Moreover, it has been speculated that often, more than one uptake pathway is possible, dependent on factors such as the peptide concentration applied, the cell line used, the cargo attached and the overall incubation conditions [14,16,17].

In our previous report, we analysed the cellular uptake of 22 CPPs in HEK293, HeLa, MDCK and Cos-7 cells [7] showing the importance of the appropriate CPP choice for a distinct application. On the basis of these results, we then selected six CPPs, namely

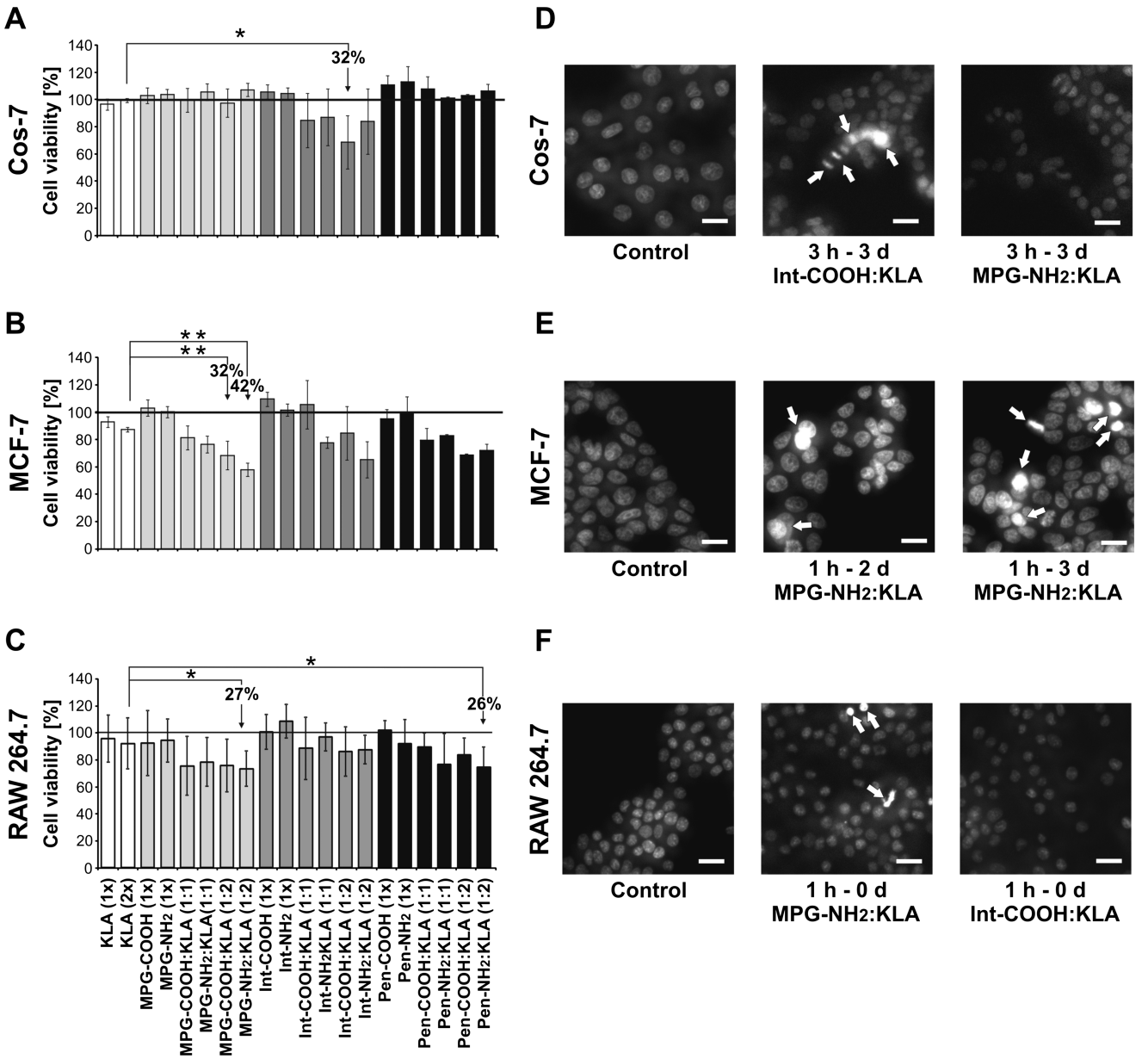

Figure 4. Activity of the pro-apoptotic KLA peptide: (A–C) Viability after CPP : KLA incubation: Cells were incubated with the CPP : KLA mixture, and the cell viability was detected using the MTT test. One hundred per cent cell viability corresponds to no toxic effect of the peptides. In Cos-7 cells, the mixture of 10 μM Int-COOH + 20 μM KLA reveals a toxic effect of 32% ($p < 0.05$). The mixture of 1 μM MPG-NH_2 + 2 μM KLA reveals a toxic effect of 42% ($p < 0.001$) for MCF-7 and of 27% ($p < 0.05\%$) for RAW 264.7. Data shown are the means ± SEM ($n = 3$). (D–E) Nuclear condensation after CPP : KLA incubation: Cells were incubated with the CPP : KLA (1 : 2) mixtures and stained with Hoechst 33342 as indicated. Arrows point to condensed chromatin. The scale bar represents 20 μm. Condensed chromatin was revealed in cells treated with the conditions that showed toxic effects in the MTT tests.

the Tat peptide, $S4_{13}PV$, R9, penetratin and integrin peptides, and MPG for further investigations. By trying to optimise their cellular uptake, we evaluated the influence of the C-terminus (-COOH vs -$CONH_2$) on the internalisation. We discovered that the carboxyamidated form heavily enhanced the ability of CPP penetration in MCF-7, RAW 264.7 and Cos-7 cells (Figure 1A) for most but not all tested CPPs. Surprisingly, the Tat peptide shows very low uptake rates independent of its C-terminus, which is in coherence with our previous results [7]. However, the cellular distribution of the CPPs was not altered in most cases when changing the C-termini (Figure 1B and Figure S1). CPPs were mostly detected in a punctuated pattern hinting towards endosomal entrapment. Only cancer cells showed different localisations; here, carboxyamidated CPPs seem to be able to escape the endosomes and even penetrate the nucleus. As reported in 2008, we confirm again that CPP internalisation depends on the used CPP and chosen cell type as well as – and this is new – on the C-terminus of the CPP itself.

In this study, our main goal was to compare the delivery of the pro-apoptotic KLA peptide in breast adenocarcinoma cells, macrophages and fibroblasts using the three best CPPs (integrin and penetratin peptides and MPG) and to prove their therapeutic applicability. Nowadays, the development and analysis of CPPs conjugated to anticancer drugs to serve as potential new chemotherapeutic agents becomes the focus of attention. In order to offer an alternative to covalent methods, we proposed a strategy

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for the delivery of the pro-apoptotic KLA peptide [9] into mammalian cells, on the basis of a simple co-incubation with the appropriate CPP.

After determining the optimal mixing ratios (1 : 1 and 1 : 2 of CPP : KLA), we clearly demonstrated a twofold to threefold enhancement of the KLA uptake (Figure 2B). Unfortunately, we did not perceive a general rule for a successful KLA delivery but learned that different cell types needed different CPPs with different C-termini to deliver the KLA peptide effectively. Furthermore, the CPP uptake did not necessarily correlate with the KLA uptake. For example, MPG-NH_2 having the highest internalisation properties in all cell lines (Figure 1A) showed only an improvement of the KLA delivery in RAW264.7 cells compared with the other employed CPP : KLA pairs in all other cell lines (Figure 2B). In contrast, the Int-COOH : KLA mixture (1 : 1 or 1 : 2) revealed the best KLA delivery in Cos-7 cells, although Int-COOH alone seemed to have nearly no penetration ability (Figure 1A). Confocal laser scanning microscopy (CLSM) confirmed the measurements done before; KLA was successfully delivered into the cells but showed mostly a punctuated pattern, which might have been because of endosomal entrapment [18]. Besides, we observed a diffuse KLA distribution and suggest that it resulted from an endosomal KLA release (Figure 3B). By simply mixing the CPP and KLA peptides, it is highly likely that we had no proper complex formation as shown for CPP : ON complexes [19] or a formation of aggregates as reported in [20]. The KLA peptide was presumably endocytosed together with the CPP. Over the past years, it became clear that CPPs are internalised at low concentrations via energy-dependent mechanisms [21]. Therefore, we assume that the internalisation of the KLA peptide was facilitated by the endocytosis of CPPs.

However, by looking on the pro-apoptotic properties of internalised KLA peptide in MTT assays (Figure 4A), we were able to depict one combination, which was toxic to macrophages and cancer cells but showed no toxicity in fibroblasts. The mixture of MPG-NH_2 : KLA (1 : 2) resulted in 42% toxicity ($p < 0.001$ vs KLA alone) in MCF-7 cells and in 36% toxicity ($p < 0.05$ vs KLA alone) in RAW 264.7 cells. This is particularly interesting because we used tenfold lower concentrations as in the incubations with Cos-7 cells. For the Cos-7 cells, only the combination of Int-COOH : KLA (1 : 2) revealed a reduction in cell viability of 32%. These results were validated when microscopically examining the nuclear condensation, which is a hallmark of apoptosis. For the conditions causing toxicity in the MTT viability measurements, we observed a distinguished nucleus condensation in all three cell lines (Figure 4D–E).

The comparison with published reports to validate our results is difficult because the internalisation is usually done by a covalent linkage of the KLA peptide to CPPs [22,23], to homing peptides [9] or to self-assembling fibres [24]. Nevertheless, in all cases, researchers used covalent-bound peptide concentrations comparable with ours (2–10 μM) or even higher (up to 100 μM). In our report, we demonstrated – that using the simple co-incubation strategy to administer the KLA peptide in a low micromolar range (1 μM CPP + 2 μM KLA) – we obtained a pro-apoptotic effect of the KLA peptide ranging between 25% and 60%, which is in the same range of induced toxicity as described in the other studies [9,22,23].

All together, our simple co-incubation strategy enabled us to introduce the pro-apoptotic KLA peptide in the carcinoma cell line MCF-7 and in the macrophagic cell line RAW264.7, both involved in cancer development and metastasis formation. For these cell lines, we reach a 36% and 42% cell viability reduction, which coincides with the effect of KLA covalently coupled to CPPs [22,23] or with KLA nanofibres [24] even though we used much shorter incubation times. More importantly, this effect was not observable in fibroblastic Cos-7 cells even at tenfold higher concentrations.

In summary, the co-incubation strategy may hold many benefits such as the combinatorial potential of different CPPs with different pro-apoptotic peptides, the use of targeting devices and associated cost-savings because of shorter peptide sequence. These advantages are highly relevant in regard to a therapeutic application of pro-apoptotic peptides in cancer treatment. In the case of an occurring resistance to one therapeutic peptide, it can be quickly and easily replaced when the mixture is applied.

Conclusion

This approach represents a huge benefit for the future therapeutic utilisation: (i) It is much easier and quicker to co-incubate the appropriate CPP with the corresponding therapeutic peptide instead of newly synthesising the chosen set, and (ii) It is possible to change the pro-apoptotic peptide quickly in case resistance occurs.

These results show the difficulty that scientists have when working with CPPs. Which CPP is the 'best' one for the application? We can only give the advice to compare different CPPs in the respective cell line taking the C-terminus into account. This is aggravated by the fact that a chosen CPP might be perfect in its toxicity and uptake properties but might not show the expected transportation capabilities. Therefore, we recommend including always more than one CPP in the evaluation process.

Experimental Section

Peptide Synthesis

The peptides used for the binding studies were automatically synthesised (Syro II, MultiSynTech, Witten, Germany) using to the Fmoc chemistry. Peptides with a C-terminal carboxyl group were generated using TentaGel S PHB-aa-Fmoc (Rapp Polymere, Tuebingen, Germany) and with a C-terminal carboxyamide group using TentaGel S Ram resin (Rapp Polymere, Tuebingen, Germany). The crude peptides were purified to >95% using preparative HPLC (Waters, Eschborn, Germany), and their identity was determined using analytical RP-HPLC (Waters, Eschborn, Germany) and MALDI TOF mass spectrometry (LaserTec BenchTopII, PerSeptive Biosystems, Carlsbad, California, US).

Cell Culture

African green monkey kidney cells (Cos-7) were cultured as described in [7]. Human breast adenocarcinoma cells (MCF-7) were cultured in phenol red-free (prf) RPMI 1640 (Invitrogen, Karsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Karsruhe, Germany), non-essential amino acids (MEM NEAA, Gibco, Karsruhe, Germany), 1 mM sodium pyruvat (Gibco, Karlsruhe, Germany) and 1% penicillin/streptomycin (pen/strep, Biochrom AG, Berlin, Germany). Mouse leukaemic macrophages (RAW 264.7) were cultured in RPMI 1640 (prf), 10% heat-inactivated FBS, 0.05 mM-glutamine (Gibco, Karlsruhe, Germany) and 1% pen/strep. The cells were seeded 1×10^5 cells/ml in 1 ml medium in 12-well culture plates (Falcon, BD Biosciences, Heidelberg, Germany) and grown until ~80% confluence at the day of the experiment. Unless

otherwise indicated, solutions were tempered to 37 °C, and all incubations were performed at 37 °C, 5% CO_2.

Cellular Uptake

Uptake measurements were performed as reported previously [5]. In detail, the cells were rinsed twice with phosphate buffered saline and incubated with 500 µl of a 10 µM peptide solution (in FBS-free medium) for 30 min. The CPP concentrations were determined via the peptides' CF labels (excitation, 485 nm; emission, 520 nm). The KLA concentration in the lysate was measured via the peptides' Cy5-labels (excitation, 640 nm; emission, 680 nm). If not otherwise indicated, the CPPs and KLA peptide were directly co-incubated with the cells and not pre-incubated. For both conditions, the results were normalised to the total protein content using a BCA (bicinchoninic acid) protein assay.

Confocal Laser Scanning Microscopy

Confocal images were performed as previously described [5]. Briefly, cells were seeded in glass-bottom dishes (World Precision Instruments) with an appropriate density of ~1×10^5 cells/well 1 day before the experiment to achieve a maximum confluence of 80%. Confocal images were acquired with an inverted IX81 fluorescence microscope (Olympus, Teltow, Germany).

To detect the KLA delivery by CPPs, the KLA peptide was labelled with Cy5. Cells were incubated with KLA and CPPs at respective concentrations (Cos-7: 10 µM integrin peptide + 10/20 µM KLA; MCF-7: 1 µM integrin peptide + 1/2 µM KLA; RAW 264.7: 1 µM MPG + 1/2 µM KLA) for 30 min, washed and analysed in complete growth medium. Hoechst 33342 (Molecular Probes, Saint Aubin, France) was used to stain nuclei. The pictures were then obtained using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Le Pecq, France). All images were performed using living, non-fixed cells.

For nuclear morphology analysis, cells (8×10^4 cells per dish) were seeded and the next day incubated with indicated CPP : KLA complexes. Directly after or after 24 or 48 h, the cells were stained with 10 µg/ml Hoechst 33342 (Molecular Probes, Saint Aubin, France) for 30 min. The nuclear morphology was analysed with a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Le Pecq, France).

MTT Assay

A good cellular tolerance is highly important for the use of CPPs as delivery reagents. Cell viability was determined using the colorimetric MTT dye as presented in [5]. In a few words, to test the cytotoxicity of CPPs, cells were incubated with peptides for 2 h in FBS-free medium, washed and further incubated with the appropriate medium containing FBS and the MTT dye for 4 h. The absorbance at 570 nm was then measured. The results were normalised to the control (without peptide) that corresponds to 100% viability.

Measurements of the KLA peptide activity were performed using the MTT test as well. Therefore, cells were incubated with CPPs (10 µM for Cos-7, 1 µM for MCF-7 and RAW 264.7) and KLA (10 or 20 µM for Cos-7, 1 or 2 µM for MCF-7 and RAW 264.7) alone or as a mixture. The incubation time was 3 h for Cos-7 cells and 1 h for MCF-7 and RAW 264.7 cells. The MTT test was performed after 3 days for Cos-7 and MCF-7 cells and after different time points (0–3 days) for RAW 264.7 cells. The results were normalised to the control (without peptide) that corresponds to 100% viability.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Multiple comparisons between groups were assessed using one-way analysis of variance with Newman–Keuls *post hoc* test. Probability values <0.05 were accepted as statistically significant and the p values were noted as follows: p = not significant for $p > 0.05$ and * for $p < 0.05$, ** for $p < 0.001$ and *** for $p < 0.0001$. Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

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