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Enzyme-triggered delivery of chlorambucil from conjugates based on the cell-penetrating peptide BP16

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The undecapeptide KKLFKKILKKL-NH_2 (**BP16**) is a non-toxic cell-penetrating peptide (CPP) that mainly internalizes cancer cells through a clathrin dependent endocytic mechanism and localizes in late endosomes. Moreover, this CPP is able to enhance the cellular uptake of chlorambucil (CLB) improving its cytotoxicity. In this work, we further explored the cell-penetrating properties of **BP16** and those of its arginine analogue **BP308**. We investigated the influence on the cytotoxicity and on the cellular uptake of conjugating CLB at the N- or the C-terminal end of these undecapeptides. The effect of incorporating the cathepsin B-cleavable sequence Gly-Phe-Leu-Gly in CLB-BP16 and in CLB-BP308 conjugates was also evaluated. The activity of CLB was significantly improved when conjugated at the N- or the C-terminus of **BP16**, or at the N-terminus of **BP308**. While CLB alone was not active (IC_{50} of 73.7 to >100 μM), the resulting conjugates displayed cytotoxic activity against CAPAN-1, MCF-7, PC-3, 1BR3G and SKMEL-28 cell lines with IC_{50} values ranging from 8.7 to 25.5 μM. These results were consistent with the internalization properties observed for the corresponding 5(6)-carboxyfluorescein-labeled conjugates. The presence of the tetrapeptide Gly-Phe-Leu-Gly at either the N- or the C-terminus of CLB-BP16 conjugates further increased the efficacy of CLB (IC_{50} of 3.6 to 16.2 μM), which could be attributed to its selective release in the lysosomal compartment. Enzymatic assays with cathepsin B showed the release of CLB-Gly-OH from these sequences within a short time. Therefore, the combination of BP16 with an enzymatic cleavable sequence can be used as a drug delivery system for the effective uptake and release of drugs in cancer cells.

Introduction

In recent years, drug delivery systems have been extensively studied in an effort to improve cancer treatments.[1,2] The use of well-known anticancer drugs is usually hindered by their poor bioavailability and their low capacity to cross cell membranes.[3] One strategy to overcome these limitations is their conjugation to a convenient carrier able to promote an efficient cell uptake of the drug. Cell-penetrating peptides (CPPs) have been established as peptide-based delivery systems to transport a wide range of cell-membrane impermeable cargoes into the cells.[4-11] CPPs have been shown to increase the bioavailability of anticancer drugs, improving their cytotoxic activity while dramatically reducing their dosage and side-effects.[12-16]

The exact internalization pathway of CPPs is still under debate. However, it is widely assumed that their high positive charge facilitates their interaction with the negatively charged plasma membrane and that they are mainly internalized by endocytosis.[17,18] Since following this endocytic pathway CPPs generally end up in the lysosomal compartment of cells, enzyme-triggered strategies are recently being used in smart drug delivery systems to improve intracellular drug release.[19,20] Lysosomal proteases are expected to selectively cleave and release active drug moieties within the cell.[21] This proteolytic activity depends on the pH gradients occurring at the subcellular level and allows an intracellular-specific delivery.[22-27] The tetrapeptide Gly-Phe-Leu-Gly is a well-known cleavable sequence, which is selectively hydrolyzed by cathepsin B cysteine protease, overexpressed in lysosomes.[21,28-30] Indeed, this sequence has been employed in several drug-peptide conjugation strategies, leading to a selective intracellular drug release and enhancing its cytotoxic activity.[31-34]

Recently, we identified the undecapeptide KKLFKKILKKL-NH_2 (**BP16**) as a non-cytotoxic CPP with high cellular uptake *in vitro*.[35] We demonstrated that **BP16** is mainly internalized in MCF-7 cells through clathrin dependent endocytosis and that efficiently accumulates in the cell

Table 1 Peptide sequences and notation

Peptide	Sequence[a]	Notation
BP16	KKLFKKILKKL	
BP308	RRLFRRILRRL	
BP325	CLB-KKLFKKILKKL	CLB-BP16
BP331	Ac-KKLFKKILKKLK(CLB)	BP16-CLB
BP332	CLB-GFLG-KKLFKKILKKL	CLB-GFLG-BP16
BP333	Ac-KKLFKKILKKLK(GLFG-CLB)	BP16-GLFG-CLB
BP334	CLB-RRLFRRILRRL	CLB-BP308
BP335	Ac-RRLFRRILRRLK(CLB)	BP308-CLB
BP336	CLB-GFLG-RRLFRRILRRL	CLB-GFLG-BP308
BP337	Ac-RRLFRRILRRLK(GLFG-CLB)	BP308-GLFG-CLB
CF-BP16	CF-KKLFKKILKKL	CF-BP16
CF-BP308	CF-RRLFRRILRRL	CF-BP308
BP326	CLB-KKLFKKILK(CF)KL	CLB-BP16-CF
BP338	CF-KKLFKKILKKLK(CLB)	CF-BP16-CLB
BP339	CLB-RRLFRRILRRLK(CF)	CLB-BP308-CF
BP340	CF-RRLFRRILRRLK(CLB)	CF-BP308-CLB

[a]All peptides are C-terminal amides.

endosomes. **BP16** displayed high efficient drug delivery properties since it was able to enhance the uptake of the DNA alkylating drug chlorambucil (CLB), improving its cytotoxicity against cancer cells between 6 to 9-fold. In conjugation with the homing peptide CREKA, **BP16** was also able to internalize CLB increasing its cytotoxic effect from 2- to 4.5-fold.

These findings prompted us to further explore the CPP properties of **BP16**. With this aim, we investigated the influence of conjugating CLB at the N- or at the C-terminus of **BP16** on the cellular uptake and on the cytotoxic activity against cancer cells. Moreover, taking into account the clathrin dependent endocytic mechanism of **BP16**, we envisaged that the introduction of the cleavable sequence Gly-Phe-Leu-Gly in CLB-BP16 conjugates could result in an efficient release of the drug. Thus, we evaluated the effect on the cytotoxicity of incorporating this tetrapeptide at the N- or at the C-terminal end of **BP16**. A similar study was planned with the BP16-arginine analogue **BP308** (RRLFRRILRRL-NH_2).[35] This undecapeptide was previously identified and also displayed no cytotoxicity against both malignant and non-malignant cells and, therefore, **BP308** showed a suitable activity profile to be considered as a CPP candidate.[36] Moreover, it has been recently reported that the replacement of Lys by Arg in *KLA* antimicrobial peptide led to an enhancement of the peptide accumulation in mitochondria.[37] Thus, **BP308** could favor the selective delivery of CLB into the mitochondria. The internalization of **BP16** and **BP308** was examined by flow cytometry and confocal microscopy studies were performed in order to determine their final localization at the subcellular level. The cytotoxicity of the **BP16** and **BP308** CLB-conjugates was evaluated against cancer and healthy cell lines and the correlation with their cellular uptake was analyzed. In addition, the CLB release from BP16 conjugates incorporating the tetrapeptide Gly-Phe-Leu-Gly was evaluated by cathepsin B enzymatic digestion.

Results and discussion

Peptide design and synthesis

During previous studies, **BP16** and **BP308** were identified from a library of antimicrobial undecapeptides as potential CPP candidates.[35] Both peptides were neither cytotoxic nor hemolytic. **BP16** was highly internalized in cancer cells and was able to transport CLB into these cells enhancing its efficacy.

Based on this, we decided to get a further insight into the cell-penetrating properties of **BP16** and **BP308**. In particular, in this study we investigated: (i) the influence of the position of CLB in the peptide sequence on the cytotoxicity and on the cellular uptake, and (ii) if the incorporation of the cathepsin B-cleavable sequence Gly-Phe-Leu-Gly in CLB-peptide conjugates could provide the intracellular release of the drug. Towards these aims, we designed two sets of peptide conjugates. For a list of the full set of peptides prepared in this work see Table 1. The first set incorporated CLB at the N- or at the C-terminus of **BP16** and **BP308**. The conjugates derived from **BP16** were CLB-BP16 (**BP325**) and BP16-CLB (**BP331**), and those derived from **BP308** were CLB-BP308 (**BP334**) and BP308-CLB (**BP335**). To analyze the cellular internalization of these peptides, they were labeled with 5(6)-carboxyfluorescein leading to **CF-BP16**, CLB-BP16-CF (**BP326**), CF-BP16-CLB (**BP338**), **CF-BP308**, CLB-BP308-CF (**BP339**), and CF-BP308-CLB (**BP340**). The second set of peptides was designed by incorporating CLB-Gly-Phe-Leu-Gly at the N- or at the C-terminus of **BP16** and **BP308**. Thus, this set included CLB-GFLG-BP16 (**BP332**), BP16-GLFG-CLB (**BP333**), CLB-GFLG-BP308 (**BP336**) and BP308-GLFG-CLB (**BP337**). For the conjugates that contain CLB or GLFG-CLB at the C-terminus, these moieties were incorporated at the side-chain of an additional Lys residue. This amino acid was included to allow the introduction of these moieties without affecting the cationic charges of the corresponding parent sequence.

Peptide conjugates were manually prepared on solid-phase following a standard Fmoc/*t*Bu strategy and were obtained as C-terminal amides. The synthesis of the N-terminal CLB-peptide conjugates **BP325** (CLB-BP16) and **BP334** (CLB-BP308) involved the preparation of the peptidyl resins Fmoc-Lys(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-Leu-Rink-MBHA (**1**) and Fmoc-Arg(Pmc)-Arg(Pmc)-Leu-Phe-Arg(Pmc)-Arg(Pmc)-Ile-Leu-Arg(Pmc)-Arg(Pmc)-Leu-Rink-MBHA (**2**), respectively (Scheme 1). They were obtained from an Fmoc-Rink-MBHA resin through sequential Fmoc removal and amino acid coupling steps. The Fmoc group was removed by treatment with a piperidine/DMF (3:7) solution. Couplings of the conveniently protected Fmoc amino acids were mediated by *N,N'*-diisopropylcarbodiimide (DIPCDI) and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) in *N,N*-dimethylformamide (DMF). Once the synthesis was completed, peptidyl resins **1** and **2** were treated with piperidine/DMF (3:7) and CLB was coupled by using DIPCDI and Oxyma in DMF. Acidolytic

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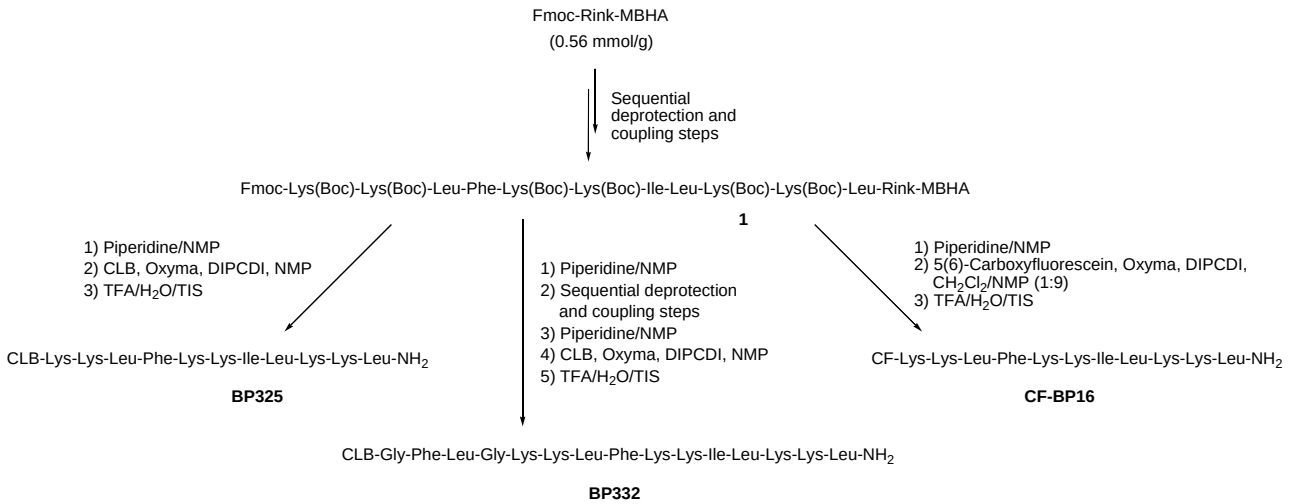
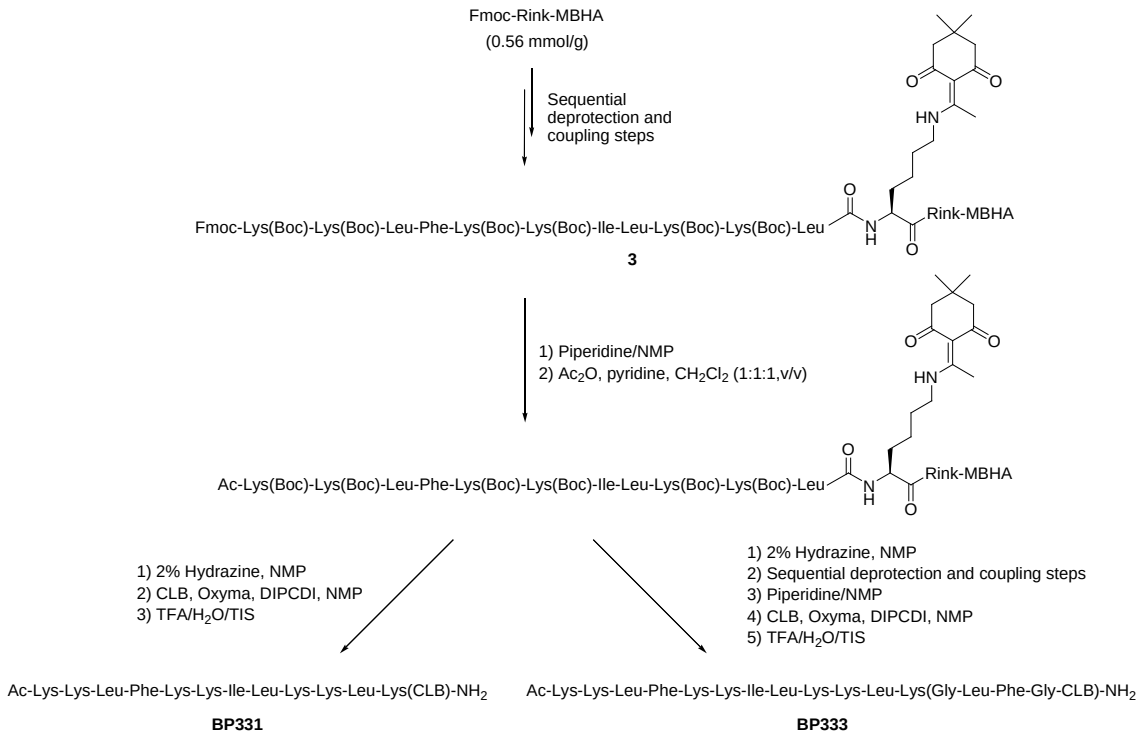


Scheme 1 Synthesis of the conjugates **BP325** and **BP332** and of the fluorescently labeled peptide **CF-BP16**



Scheme 2 Synthesis of peptide conjugates **BP331** and **BP333**

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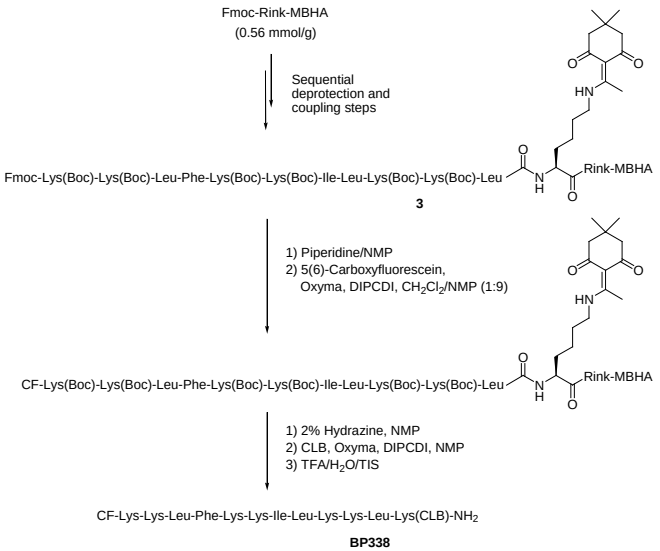

Scheme 3 Synthesis of fluorescently labeled conjugate **BP338**

cleavage with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O (95:2.5:2.5) afforded peptide conjugates **BP325** and **BP334** in 91 and >99% purity, respectively. Peptide conjugates **BP332** (CLB-GFLG-BP16) and **BP336** (CLB-GFLG-BP308), incorporating CLB-GFLG at the N-terminus of **BP16** or **BP308**, were prepared from peptidyl resins **1** and **2**, respectively. After removal of the N-terminal Fmoc group, the corresponding Fmoc-protected amino acids of the GFLG moiety and CLB were sequentially incorporated. Acidolytic treatment of the resulting resins yielded **BP332** and **BP336** in 92 and >99% purity, respectively. All these peptides were characterized by HRMS.

The synthesis of peptide conjugates **BP331** (BP16-CLB), **BP333** (BP16-GLFG-CLB), **BP335** (BP308-CLB) and **BP337** (BP308-GLFG-CLB), bearing CLB or GLFG-CLB at the C-terminus of **BP16** or **BP308** is depicted in Scheme 2 for the **BP16** derivatives. These moieties were incorporated at the side-chain of a Lys residue present at the C-terminal end of the peptide. To allow its selective derivatization, this Lys residue was incorporated as Fmoc-Lys(Dde)-OH (Dde = *N*-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]). Thus, peptidyl resins Fmoc-Lys(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-Leu-Lys(Dde)-Rink-MBHA (**3**) and Fmoc-Arg(Pmc)-Arg(Pmc)-Leu-Phe-Arg(Pmc)-Arg(Pmc)-Ile-Leu-Arg(Pmc)-Arg(Pmc)-Leu-Lys(Dde)-Rink-MBHA (**4**) were prepared. After deprotection and acetylation of the N-terminus, the Dde group was selectively removed by treatment with hydrazine. In the case of **BP331** and **BP335**, the resulting free amino group was acylated with CLB. For **BP333** and **BP337**, the corresponding amino acids of the GLFG moiety and CLB were sequentially incorporated at this free amino group. Peptide conjugates were cleaved from the resin by acidolytic treatment and were obtained in excellent purities (91->99%, Table 1), as determined by analytical HPLC. Their identities were further confirmed by HRMS.

The fluorescently labeled peptides **CF-BP16** and **CF-BP308** were obtained from peptidyl resins **1** and **2**, respectively, by Fmoc removal and coupling of 5(6)-carboxyfluorescein (CF) using DIPCDI and Oxyma (Scheme 1). Piperidine washes were performed before cleavage of the peptide from the resin which served to remove overincorporated carboxyfluorescein moieties.[39] The CF-labeled peptides **BP338** (CF-BP16-CLB) and **BP340** (CF-B308-CLB), bearing the carboxyfluorescein at the N-terminus and the CLB at the C-terminus, were prepared from peptidyl resins **3** and **4**, respectively (Scheme 3). The synthesis consisted in deprotection of the N-terminus, carboxyfluorescein coupling, Dde group removal and acylation with CLB. The preparation of the CF-labeled peptides **BP326** (CLB-BP16-CF) and **BP339** (CLB-BP308-CF), incorporating a CLB moiety at the N-terminus and a carboxyfluorescein group at the C-terminus involved the synthesis of peptidyl resins Fmoc-Lys(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Leu-Lys(Dde)-Lys(Boc)-Leu-Rink-MBHA and **4**, respectively. Fmoc removal and N^{α}-amino group acylation with CLB was followed by Dde group removal and derivatization with 5(6)-carboxyfluorescein. Acidolytic cleavage afforded the CF-labeled peptides in excellent purities (81->99%), and they were characterized by HRMS.

Cellular uptake of CF-BP16 and CF-BP308

In order to evaluate the influence of replacing Lys residues with Arg on the cellular uptake of **CF-BP16**, we first examined the capacity of internalization of **CF-BP308** into MCF-7 cells by flow cytometry and compared it to that of **CF-BP16**. MCF-7 cells were incubated with **CF-BP308** or with **CF-BP16** at 25 µM for different times (1, 3 and 6 h) at 37 ºC. Cells were harvested by trypsinization, which also prevented non-specific plasma membrane binding of the peptide. As depicted in Figure 1, the mean intracellular fluorescence of the cells incubated with either **CF-BP308** or **CF-BP16** increased over time. No significant differences were observed in the mean fluorescence intensity values for both peptides (1065 ± 44 and 981 ± 159 after 6 h, respectively).

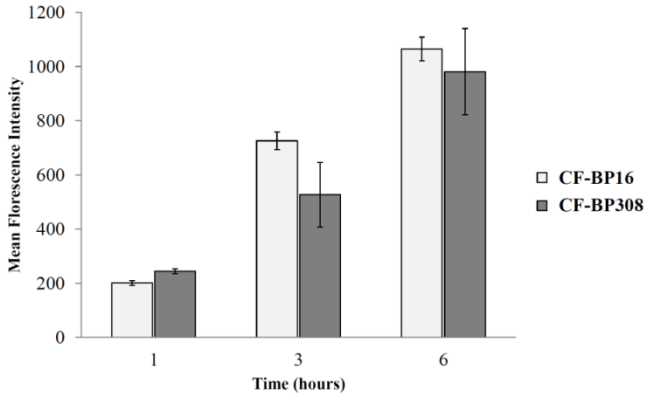

Fig. 1 Comparison of the cellular uptake between **CF-BP16** and **CF-BP308** in MCF-7 cells. Cells were exposed to the peptide at 25 µM for 1, 3 and 6 h at 37 ºC. Each column in the graph represents the mean fluorescence intensity of the cells determined in three independent experiments ± SD.

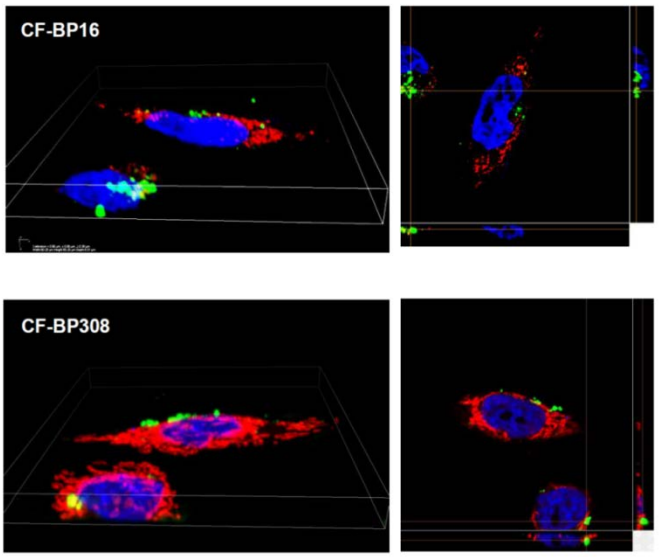

Fig. 2 Confocal microscopic imaging of the internalization of **CF-BP16** and **CF-BP308** into MCF-7 cells. Cells were incubated with **CF-BP16** and **CF-BP308** at 50 µM for 30 min at 4 ºC and for 3 h at 37 ºC. Cell nuclei were stained with Hoechst (blue) and the mitochondria were stained with Mitotracker Red (red). The localization of peptides is indicated by the green fluorescence. The images show the 3D reconstruction of the confocal Z stack images.

Confocal studies were carried out to examine if the internalization in MCF-7 cells of the arginine analogue **CF-BP308** correlated with that of **CF-BP16**. In a previous study we observed that **CF-BP16** accumulates in vesicles throughout the cytoplasm of these cells, with significant clustering at the periphery of the nucleus.[35] An optical sectioning indicated that no fluorescence particles were placed inside the nucleus. Moreover, it has been described that the replacement of D-Lys by D-Arg in the *KLA* antimicrobial peptide led to an enhancement of the mitochondrial accumulation in HeLa cells.[37] Thus, further confocal microscopy studies were performed in order to find out if these peptides colocalize with mitochondrial markers.

To achieve these aims, MCF-7 cells were incubated with the corresponding CF-labeled peptide at 50 µM for 30 min at 4 ºC and for 3 h at 37 ºC. The cell nuclei and mitochondria were stained with Hoechst and Mitotracker Red, respectively (Figure 2). As expected, **CF-BP308** showed the same cellular uptake behavior as **CF-BP16** and no fluorescence was observed after 30 min of incubation at 4 ºC, demonstrating that both CF-labeled peptides internalize through energy-dependent mechanisms.[35] The number of fluorescent particles gradually increased over time and a dot-like distribution pattern was observed for both peptides after 3 h of incubation. Similarly to **CF-BP16**, higher-magnification (1000×) images from MCF-7 cells incubated with **CF-BP308** revealed that the fluorescent particles were located throughout the cytoplasm and that no colocalization was observed in the nucleus (Figure 2). Moreover, as shown by optical sectioning, neither **CF-BP16** nor **CF-BP308** accumulated inside the mitochondria, pointing out that these peptides do not show mitochondria targeting properties.

Although **CF-BP16** and **CF-BP308** did not colocalize with the nucleus or mitochondria, these results indicate that both peptides display potential internalization properties for being used in drug delivery systems. In particular, since previous mechanistic studies on the cellular uptake of **BP16** suggested its localization in late endosomes,[35] these peptides could be useful in site-specific release strategies occurring at the lysosomal compartment.[21]

Cell cytotoxicity of the CLB-peptide conjugates

The cytotoxic activity profile of the CLB-peptide conjugates was explored to determine the influence of incorporating CLB at the N- or at the C-terminus of the peptide as well as of the presence of the Gly-Phe-Leu-Gly enzymatic cleavable sequence. Thus, MCF-7, CAPAN-1, PC-3, SKMEL-28 and 1BR3G cells were exposed to the CLB-peptide conjugates, and the IC_{50} was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 48 h of peptide exposure. CLB was included in the study for comparison purposes.

As shown in Table 2, except for **BP335**, **BP336** and **BP337**, peptide conjugates were active against the cancer and healthy cell lines tested. In contrast, CLB was not cytotoxic against the five cell lines (IC_{50} of 73.7 to >100 µM). Notably, conjugation of CLB at the N- or at the C-terminus of **BP16** resulted in peptides **BP325** (CLB-BP16) and **BP331** (BP16-CLB), respectively, with important activity (IC_{50} of 8.7 to 25.5 µM). No significant differences were observed between the cytotoxicity of these two peptide conjugates against CAPAN-1 and PC-3 cells. **BP325** was more active than **BP331** against MCF-7 and 1BR3G cells, but less active against SKMEL-28 cells. A further improvement of the activity of CLB was achieved when the tetrapeptide Gly-Phe-Leu-Gly was incorporated between CLB and the N- or the C-terminus of **BP16**. Thus, **BP332** (CLB-GFLG-BP16) exhibited higher cytotoxicity than **BP325** (CLB-BP16), being 2- to 4-fold more active against CAPAN-1, PC-3 and SKMEL-28 cells (IC_{50} of

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Table 2 Cytotoxicity (IC_{50}) of CLB and CLB-peptide conjugates in CAPAN-1, MCF-7, PC-3, SKMEL-28 and 1BR3G cells[a]

Peptide	Notation	CAPAN-1	MCF-7	PC-3	SKMEL-28	1BR3G
CLB		> 100	73.7 ± 4.5	> 100	> 100	> 100
BP325	CLB-BP16	13.7 ± 2.4	12.0 ± 2.7	11.3 ± 3.2	15.0 ± 3.0	18.2 ± 3.5
BP331	BP16-CLB	11.2 ± 2.5	25.5 ± 0.7	12.9 ± 2.0	8.7 ±3.4	24.0 ± 1.3
BP332	CLB-GFLG-BP16	7.2 ± 0.3	14.0 ± 1.4	3.6 ± 0.4	3.9 ± 1.5	14.3 ± 0.6
BP333	BP16-GLFG-CLB	6.2 ± 0.3	16.2 ± 1.8	8.2 ± 0.5	6.4 ± 2.3	15.7 ± 0.6
BP334	CLB-BP308	11.2 ± 1.9	15.0 ± 5.0	16.4 ± 3.4	12.8 ± 2.9	13.8 ± 3.4
BP335	BP308-CLB	> 100	> 100	> 100	> 100	> 100
BP336	CLB-GFLG-BP308	> 100	> 100	> 100	> 100	> 100
BP337	BP308-GLFG-CLB	> 100	> 100	> 100	> 100	> 100

[a]The IC_{50} values were determined by the MTT assay after 48 h of CLB-peptide conjugate exposure. Data represent the mean ± SD of at least three independent experiments performed in triplicate.

7.2, 3.6 and 3.9 µM, respectively). **BP333** (BP16-GLFG-CLB) was around 1.5-fold more active against all cell lines compared to **BP331** (BP16-CLB) (IC_{50} of 6.2 to 16.2 µM vs 8.7 to 25.5 µM). These results revealed that the efficacy of CLB is enhanced when conjugated to either the N- or the C-terminal end of **BP16**. Remarkably, the introduction of the Gly-Phe-Leu-Gly enzymatic cleavable sequence further increased this efficacy irrespective of the peptide end into which is incorporated. The presence of this tetrapeptide might provide a specific recognition site for cathepsin B which could induce the release of CLB, enhancing its cytotoxicity. As previously reported, **BP16** is mainly internalized into cancer cells by clathrin dependent endocytosis and might follow a degradation pathway, where the fusion between late endosomes and lysosomes occurs. Therefore, the release of CLB from the sequences containing Gly-Phe-Leu-Gly could take place in the lysosomal compartments, which are the final stage of this endocytic pathway.

A different cytotoxic activity profile was observed for CLB-peptide conjugates derived from **BP308**. The activity of CLB was only increased when it was introduced at the N-terminus of this peptide. **BP334** (CLB-BP308) displayed IC_{50} values ranging from 11.2 to 16.4 µM against the cell lines tested, whereas **BP335** (BP308-CLB) was not active (IC_{50} >100 µM). Unlike in the case of **BP16**, the attachment of the tetrapeptide Gly-Phe-Leu-Gly to the N- or the C-terminal end of **BP308** did not improve the activity of CLB, being **BP336** (CLB-GFLG-BP308) and **BP337** (BP308-GLFG-CLB) surprisingly inactive against all cell lines (IC_{50} >100 µM).

The different behaviour of CLB-conjugates derived from **BP16** and **BP308** revealed the importance of evaluating the influence of the position of the drug in the peptide sequence on the cytotoxicity. Moreover, even though **BP308** displays similar cellular uptake than **BP16**, the fact that the incorporation of Gly-Phe-Leu-Gly in **BP308** does not enhance the cytotoxic activity of CLB suggests that the replacement of Lys by Arg in **BP16** might be associated to a different cellular uptake pattern.

Cellular uptake of CLB peptide conjugates

In order to determine whether the cytotoxic activity profile exhibited by **BP325** (CLB-BP16), **BP331** (BP16-CLB), **BP334** (CLB-BP308) and **BP335** (BP308-CLB) correlated with their internalization properties, these CLB conjugates were labeled with 5(6)-carboxyfluorescein (CF) and analyzed by flow cytometry. The cellular uptake of the CF-labeled peptide conjugates **BP326** (CLB-BP16-CF), **BP338** (CF-BP16-CLB), **BP339** (CLB-BP308-CF) and **BP340** (CF-BP308-CLB) at 25 µM was determined after 6 h of incubation in MCF-7 cells at 37 ºC (Figure 3).

Results showed that the position of CLB in **BP16** did not influence the internalization properties of this peptide, since **BP326** (CLB-BP16-CF) and **BP338** (CF-BP16-CLB) displayed similar mean intracellular intensity values (1151 ± 50 vs 1100 ± 20). Notably, the internalization ability of these peptides correlated with the cytotoxic activity exhibited by the corresponding non-labeled sequences **BP325** (CLB-BP16) and **BP331** (BP16-CLB) (Table 2).

Unlike the **BP16** derivatives, the **BP308** conjugates were differentially internalized. While **BP339** (CLB-BP308-CF) exhibited high internalization capacity (1116 ± 25), comparable to that of CLB-BP16-CF (**BP326**), **BP340** bearing the CLB moiety at the C-terminus showed a low intracellular fluorescence (348 ± 20). These results were consistent with the cytotoxic activity profile observed for the corresponding non-labeled peptides **BP334** (CLB-BP308) and **BP335** (BP308-CLB). The former was active against all cell lines whereas the latter exhibited IC_{50} values >100 µM. Thus, the low cytotoxicity of **BP335** could be attributed to its low ability to cross the cell membrane.

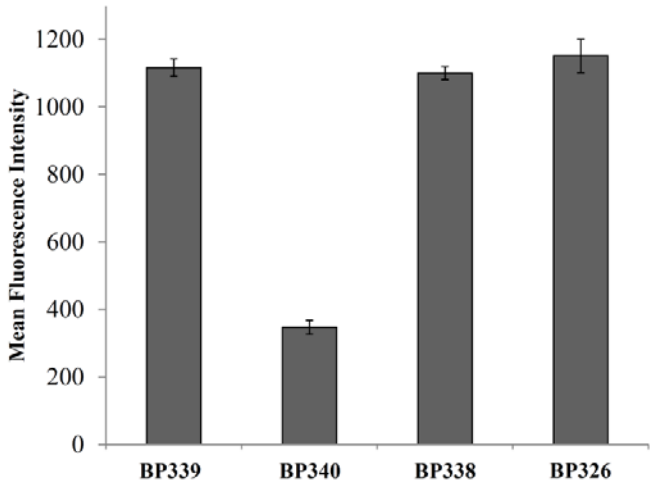

Fig. 3 Comparison of the cellular uptake of 5(6)-carboxyfluorescein labeled peptides **BP326** (CLB-BP16-CF), **BP338** (CF-BP16-CLB), **BP339** (CLB-BP308-CF) and **BP340** (CF-BP308-CLB) in MCF-7 cells. Cells were exposed to the peptide at 25 µM for 6 h at 37 ºC. Each column in the graph represents the mean fluorescence intensity of the cells determined in three independent experiments ± SD.

These studies showed that for CPPs with similar cellular uptake properties, it is important to analyse the effect of the position of the cargo in the sequence.[33,41,42] In this case, **BP16** and **BP308** have the same net charge of +6 and display comparable cell-penetrating properties in MCF-7 cells as observed by flow cytometry and confocal microscopy. However, when a cargo is attached to one of the peptide ends, the resulting **BP16** and **BP308** conjugates have a different behaviour. Whereas the uptake properties of N- and C- terminal **BP16** conjugates are similar, **BP308** derivatives display significantly different internalization capacities.

Cathepsin B enzymatic assays

To prove that the release of CLB from **BP332** (CLB-GFLG-BP16) and **BP333** (BP16-GLFG-CLB) might occur in the lysosomes, the hydrolysis of these sequences by cathepsin B was evaluated. Conditions were chosen to mimic the lysosomal medium and were based on a previously reported method.[32] The peptide conjugate **BP332** was exposed to a solution of cathepsin B in phosphate buffer (pH 5) containing 25 mM L-Cys and 1 mM EDTA. The digestion after 10, 30, 60, 90, 120 and 180 min was monitored by HPLC-MS (Figure 4A). Just after adding **BP332** to the enzyme solution (digestion time = 0 min), it was observed a mixture of this peptide (t_R = 6.3 min) together with low intensity peaks. After 10 min, the peak corresponding to **BP332** significantly decreased and a peak at a retention time of 5.2 min appeared. This peak increased over time becoming the major one after 90 min of digestion. HPLC-MS analysis revealed that this peak corresponded to CLB-Gly-OH (*m/z* = 361.1, 363.1 $[M+H]^+$; 383.0, 385.0 $[M+Na]^+$) (Figure 4C). This result pointed out that the hydrolytic cleavage occurred between the Gly and Phe residues of the enzymatic cleavable sequence Gly-Phe-Leu-Gly. The cleavage of the C-terminal analogue **BP333** (BP16-GLFG-CLB) by cathepsin B was tested and monitored (Figure 4B) using the conditions described above for **BP332**. CLB was also released from **BP333** in a time-dependent manner and following a pattern similar to that of **BP332**. The peak corresponding to CLB-Gly-OH (t_R = 5.2 min) also increased over time during the digestion. All these data are in agreement with previous studies based on cathepsin B delivery strategies.[33,34] Moreover, they support the hypothesis that CLB might be released from **BP332** and **BP333** in the lysosomes leading to the high activity observed for these peptide conjugates in cancer cells.

Conclusions

In this work we developed an efficient CLB delivery system based on **BP16**. The design of this system combined CLB and the enzymatic cleavable tetrapeptide Gly-Phe-Leu-Gly at either the N- or the C-terminus of **BP16**. The resulting sequences **BP332** and **BP333** exhibited high cytotoxic activity against cancer cells which could be attributed to the enzymatic release of CLB in the lysosomes. These results reveal that this delivery system could be applied to transport and efficiently release therapeutic agents in cancer treatments.

Experimental section

Peptide synthesis

General method for solid-phase peptide synthesis. All peptides (Table 1) were synthesized manually by the solid-phase method using Fmoc-type chemistry and the following side-chain protecting groups: *tert*-butyloxycarbonyl (Boc) for Lys and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg. A Fmoc-Rink-MBHA resin (0.56 mmol g^{-1}) was used as solid support to obtain peptide amides. Couplings of the Fmoc-amino acids (4 equiv) were performed using DIPCDI (4 equiv) and Oxyma (4 equiv) in DMF under stirring at room temperature for 2 h, and monitored by the Kaiser test.[38] For sequences containing up to eleven residues, the Fmoc group was removed by treating the resin with a mixture of piperidine/DMF (3:7, 2 + 10 min). For longer sequences, Fmoc group removal was carried out with piperidine/NMP (3:7, 1 × 3 min + 3 × 10 min). After each coupling and deprotection step, the resin was washed with DMF (6 × 1 min) and CH_2Cl_2 (6 × 1 min), and air-dried. After the coupling of the eleventh residue, NMP was used instead of DMF. Peptide elongation was performed by repeated cycles of Fmoc group removal, coupling and washings. Once the synthesis was completed, peptidyl resins were subjected to N-terminal Fmoc group removal. Then, peptides were cleaved or the peptidyl resins were derivatized with CLB, with 5(6)-carboxyfluorescein or with an acetyl group. Cleavage of peptides from the resin was performed by treatment with TFA/TIS/H_2O (95:2.5:2.5) for 3 h at room temperature, followed by TFA evaporation by bubbling N_2 into the solution. Crude peptides were precipitated by adding cold diethyl ether (-20 ºC) and collected by centrifugation. This procedure was repeated twice. Finally, peptides were dissolved in H_2O/CH_3CN (50:50 v/v containing 0.1% TFA), lyophilized

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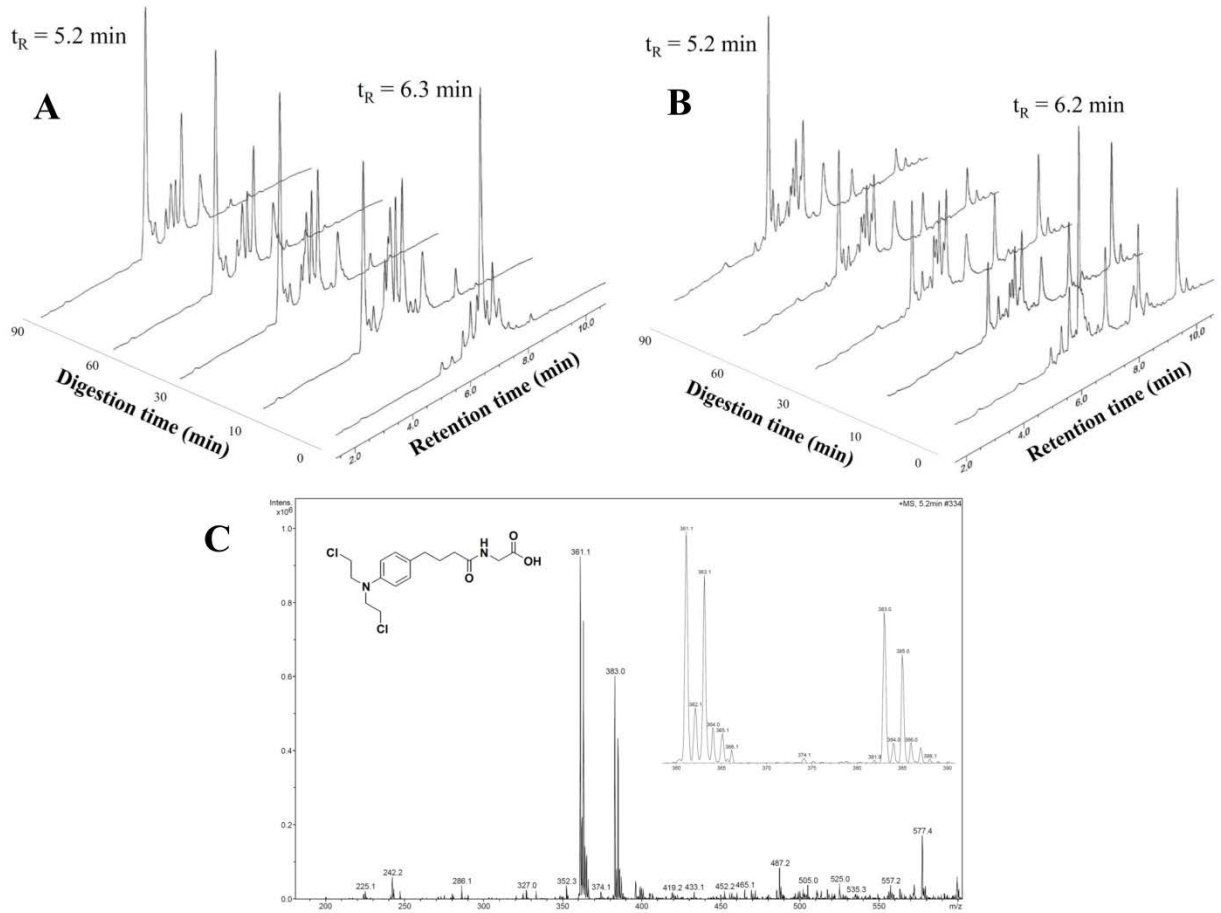

Fig. 4 Enzymatic digestion of **BP332** and **BP333** by cathepsin B monitored by HPLC-MS. A) HPLC traces of the enzymatic digestion of **BP332** after 0, 10, 30, 60 and 90 min of enzyme exposure; B) HPLC traces of the enzymatic digestion of **BP333** at 0, 10, 30, 60 and 90 min of enzyme exposure; C) ESI-MS spectrum of the peak at t_R = 5.2 min after 1 h of enzymatic digestion.

and tested for purity by HPLC. Detection was performed at 220 nm. Solvent A was 0.1% aq. TFA and solvent B was 0.1% TFA in CH_3CN. Analysis was carried out with a Kromasil 100 C_{18} (4.6 mm × 40 mm, 3.5 µm) column with a 2-100% B linear gradient over 7 min at a flow rate of 1.0 mL min^{-1}. ESI-MS and HRMS (ESI) were used to confirm peptide identity. Peptides were obtained in purities ranging from 81 to >99%.

Synthesis of the N-terminal CLB-peptide conjugates BP325, BP332, BP334, and BP336

These peptide conjugates were prepared from peptidyl resins Fmoc-Lys(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-Leu-Rink-MBHA (**1**) and Fmoc-Arg(Pmc)-Arg(Pmc)-Leu-Phe-Arg(Pmc)-Arg(Pmc)-Ile-Leu-Arg(Pmc)-Arg(Pmc)-Leu-Rink-MBHA (**2**). For the synthesis of **BP325** and **BP334**, after Fmoc removal and washes, the corresponding peptidyl resin was treated with CLB (5 equiv), DIPCDI (5 equiv) and Oxyma (5 equiv) in DMF or NMP under stirring at room temperature for 5 h. The completion of the reaction was checked by the Kaiser test.[38] The resin was then washed with NMP (6 × 1 min), CH_3OH (6 × 1 min), and CH_2Cl_2 (6 × 1 min), and air dried. To obtain **BP332** and **BP336**, after Fmoc removal and washes, the amino acids of the Gly-Leu-Phe-Gly moiety and CLB were sequentially incorporated.

CLB-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Lys-Leu-NH_2 (BP325). t_R = 7.71 min (91% purity). HRMS (ESI): *m/z* calcd. for $C_{83}H_{150}Cl_2N_{19}O_{12}$ $[M + 5H]^{5+}$ 335.4208; found 335.4228;

calcd. for $C_{83}H_{149}Cl_2N_{19}O_{12}$ $[M + 4H]^{4+}$ 418.5247; found 418.5260; calcd. for $C_{83}H_{148}Cl_2N_{19}O_{12}$ $[M + 3H]^{3+}$ 558.3631; found 558.3644; calcd. for $C_{83}H_{147}Cl_2N_{19}O_{12}$ $[M + 2H]^{2+}$ 836.0421; found 836.0404.

CLB-Gly-Phe-Leu-Gly-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Lys-Leu-NH_2 (BP332). t_R = 7.50 min (92% purity). HRMS (ESI): *m/z* calcd. for $C_{102}H_{176}Cl_2N_{23}O_{16}$ $[M + 5H]^{5+}$ 409.8603; found 409.8592; calcd. for $C_{102}H_{175}Cl_2N_{23}O_{16}$ $[M + 4H]^{4+}$ 512.0736; found 512.0709; calcd. for $C_{102}H_{174}Cl_2N_{23}O_{16}$ $[M + 3H]^{3+}$ 682.4290; found 682.4259.

CLB-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-NH_2 (BP334). t_R = 7.59 min (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{83}H_{151}Cl_2N_{31}O_{12}$ $[M + 6H]^{6+}$ 307.5256; found 307.5239; calcd. for $C_{83}H_{150}Cl_2N_{31}O_{12}$ $[M + 5H]^{5+}$ 368.6286; found 368.6283; calcd. for $C_{83}H_{149}Cl_2N_{31}O_{12}$ $[M + 4H]^{4+}$ 460.5339; found 460.5337; calcd. for $C_{83}H_{148}Cl_2N_{31}O_{12}$ $[M + 3H]^{3+}$ 613.7095; found 6131.7079.

CLB-Gly-Phe-Leu-Gly-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-NH_2 (BP336). t_R = 7.82 min (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{102}H_{177}Cl_2N_{35}O_{16}$ $[M + 6H]^{6+}$ 369.7243; found 369.7224; calcd. for $C_{102}H_{176}Cl_2N_{35}O_{16}$ $[M + 5H]^{5+}$ 443.4677; found 443.4666; calcd. for $C_{102}H_{175}Cl_2N_{35}O_{16}$ $[M + 4H]^{4+}$ 554.0828; found 554.0806; calcd. for $C_{102}H_{174}Cl_2N_{35}O_{16}$ $[M + 3H]^{3+}$ 738.4413; found 738.4394.

Synthesis of the C-terminal CLB-peptide conjugates BP331, BP333, BP335 and BP337

For the synthesis of these peptide conjugates, the Lys residue to be derivatized at the side-chain was incorporated as Fmoc-Lys(Dde)-OH (Dde = *N*-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]). **BP331** and **BP333** were prepared from the peptidyl resin Fmoc-Lys(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Leu-Lys(Boc)-Lys(Boc)-Leu-Lys(Dde)-Rink-MBHA (**3**), and **BP335** and **BP337** were prepared from Fmoc-Arg(Pmc)-Arg(Pmc)-Leu-Phe-Arg(Pmc)-Arg(Pmc)-Ile-Leu-Arg(Pmc)-Arg(Pmc)-Leu-Lys(Dde)-Rink-MBHA (**4**). After Fmoc removal and washes, the resins were treated with acetic anhydride/pyridine/CH_2Cl_2 (1:1:1, 2 × 30 min) under stirring, and washed with NMP (6 × 1 min) and CH_2Cl_2 (6 × 1 min). The completion of the reaction was checked by the Kaiser test.[38] The resulting peptidyl resins were subjected to Dde group removal by treatment with a mixture of hydrazine/NMP (2:98, 5 × 20 min). The resins were then washed with NMP (6 × 1 min) and CH_2Cl_2 (1 × 1 min). For the synthesis of **BP331** and **BP335**, the free amino group of the resulting resin was acylated with CLB as described above for the N-terminal CLB conjugates. To obtain **BP333** and **BP337**, the amino acids of the Gly-Leu-Phe-Gly moiety and CLB were sequentially incorporated at the free amino group of the corresponding resin following the protocol previously described.

Ac-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Lys-Leu-Lys(CLB)-NH_2 (BP331). t_R = 7.24 min (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{91}H_{165}Cl_2N_{21}O_{14}$ $[M + 6H]^{6+}$ 307.7032; found 307.7014; calcd. for $C_{91}H_{164}Cl_2N_{21}O_{14}$ $[M + 5H]^{5+}$ 369.0423; found 369.0408; calcd. for $C_{91}H_{163}Cl_2N_{21}O_{14}$ $[M + 4H]^{4+}$ 461.0511; found 461.0494; calcd. for $C_{91}H_{162}Cl_2N_{21}O_{14}$ $[M + 3H]^{3+}$ 614.3990; found 614.3963.

Ac-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Lys-Leu-Lys(Gly-Leu-Phe-Gly-CLB)-NH_2 (BP333). t_R = 7.38 min (91% purity). HRMS (ESI): *m/z* calcd. for $C_{110}H_{190}Cl_2N_{25}O_{18}$ $[M + 5H]^{5+}$ 443.8814; found 443.8797; calcd. for $C_{110}H_{189}Cl_2N_{25}O_{18}$ $[M + 4H]^{4+}$ 554.5999; found 554.5986; calcd. for $C_{110}H_{188}Cl_2N_{25}O_{18}$ $[M + 3H]^{3+}$ 739.1308; found 739.1295.

Ac-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-Lys(CLB)-NH_2 (BP335). t_R = 7.51 min (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{91}H_{165}Cl_2N_{33}O_{14}$ $[M + 6H]^{6+}$ 335.7093; found 335.7093; calcd. for $C_{91}H_{164}Cl_2N_{33}O_{14}$ $[M + 5H]^{5+}$ 402.6497; found 402.6502; calcd. for $C_{91}H_{163}Cl_2N_{33}O_{14}$ $[M + 4H]^{4+}$ 503.0603; found 503.0609; calcd. for $C_{91}H_{162}Cl_2N_{33}O_{14}$ $[M + 3H]^{3+}$ 670.4113; found 670.4114; calcd. for $C_{91}H_{161}Cl_2N_{33}O_{14}$ $[M + 2H]^{2+}$ 1006.1123; found 1006.1246.

Ac-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-Lys(Gly-Leu-Phe-Gly-CLB)-NH_2 (BP337). t_R = 7.67 min (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{110}H_{191}Cl_2N_{37}O_{18}$ $[M + 6H]^{6+}$ 398.0752; found 398.0749; calcd. for $C_{110}H_{190}Cl_2N_{37}O_{18}$ $[M + 5H]^{5+}$ 477.4888; found 477.4890; calcd. for $C_{110}H_{189}Cl_2N_{37}O_{18}$ $[M + 4H]^{4+}$ 596.6092; found 596.6090; calcd. for $C_{110}H_{188}Cl_2N_{37}O_{18}$ $[M + 3H]^{3+}$ 795.1431; found 795.1443.

Synthesis of the N-terminal 5(6)-carboxyfluorescein labeled peptides CF-BP16 and CF-BP308

The N-terminal labeled peptides **CF-BP16** and **CF-BP308** were prepared from peptidyl resins **1** and **2**, respectively. 5(6)-Carboxyfluorescein (2.5 equiv) was first pre-activated with Oxyma (2.5 equiv) and DIPCDI (2.5 equiv) in CH_2Cl_2/NMP (1:9) for 10 min. The mixture was added to the corresponding N-terminal deprotected peptidyl resin and reacted overnight at room temperature protected from light by covering it with aluminium foil due to the light sensitivity of the 5(6)-carboxyfluorescein. Completeness of the coupling was confirmed using the Kaiser test.[38] The resin was then washed with NMP (1 × 5 min), piperidine/NMP (1:5, 1 × 15 min), NMP (6 × 1 min), CH_2Cl_2 (6 × 1 min), CH_3OH (6 × 1 min), and CH_2Cl_2 (6 × 1 min), and air dried.[39]

CF-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Lys-Leu-NH_2 (CF-BP16). t_R = 6.76 and 6.80 min, corresponding to the two isomers of the 5(6)-carboxyfluorescein (94% purity). HRMS (ESI): *m/z* calcd. for $C_{90}H_{143}N_{18}O_{17}$ $[M + 5H]^{5+}$ 349.6170; found 349.6186; calcd. for $C_{90}H_{142}N_{18}O_{17}$ $[M + 4H]^{4+}$ 437.0203; found 437.0217; calcd. for $C_{90}H_{141}N_{18}O_{17}$ $[M + 3H]^{3+}$ 582.3579; found 582.3589.

CF-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-NH_2 (CF-BP308). t_R = 6.92 and 6.99 min, corresponding to the two isomers of the 5(6)-carboxyfluorescein (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{90}H_{144}N_{30}O_{17}$ $[M + 6H]^{6+}$ 319.5215; found 319.5195; calcd. for $C_{90}H_{143}N_{30}O_{17}$ $[M + 5H]^{5+}$ 383.2244; found 383.2234; calcd. for $C_{90}H_{142}N_{30}O_{17}$ $[M + 4H]^{4+}$ 478.7787; found 478.7778; calcd. for $C_{90}H_{141}N_{30}O_{17}$ $[M + 3H]^{3+}$ 638.0358; found 638.0341.

Synthesis of the 5(6)-carboxyfluorescein labeled CLB-peptide conjugates BP326, BP338, BP339, and BP340

BP338 and **BP340**, bearing the 5(6)-carboxyfluorescein at the N-terminus and the CLB at the C-terminus, were prepared from peptidyl resins **3** and **4**, respectively. After Fmoc removal, the resulting resins were acylated with 5(6)-carboxyfluorescein as described above for **CF-BP16** and **CF-BP308**, and treated with hydrazine/NMP (2:98, 5 × 20 min) under stirring at room temperature. Then, resins were washed with NMP (6 × 1 min) and CH_2Cl_2 (6 × 1 min), and air dried. Next, CLB coupling was carried out as described for N-terminal CLB derivatized peptides.

BP326 and **BP339**, incorporating a CLB moiety at the N-terminus and a 5(6)-carboxyfluorescein group at the C-terminus, were prepared from peptidyl resins Fmoc-Lys(Boc)-Lys(Boc)-Leu-Phe-Lys(Boc)-Lys(Boc)-Ile-Leu-Lys(Dde)-Lys(Boc)-Leu-Rink-MBHA and **4**, respectively. After Fmoc removal, the resulting resins were acylated with CLB as described above for the N-terminal CLB conjugates, and treated with hydrazine/NMP (2:98, 5 × 20 min) under stirring at room temperature. Then, resins were washed with NMP (6 × 1 min) and CH_2Cl_2 (6 × 1 min), and air dried. Next, labeling with 5(6)-carboxyfluorescein was carried out as described for the N-terminal carboxyfluorescein labeled peptides **CF-BP16** and **CF-BP308**.

CLB-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys(CF)-Lys-Leu-NH_2 (BP326). t_R = 7.56 min (81% purity). HRMS (ESI): *m/z* calcd. for $C_{104}H_{159}Cl_2N_{19}O_{18}$ $[M + 4H]^{4+}$ 508.0366; found 508.0384; calcd. for $C_{104}H_{158}Cl_2N_{19}O_{18}$ $[M + 3H]^{3+}$ 677.0464; found 677.0473; calcd. for $C_{104}H_{157}Cl_2N_{19}O_{18}$ $[M + 2H]^{2+}$ 1015.0660; found 1015.0638.

CF-Lys-Lys-Leu-Phe-Lys-Lys-Ile-Leu-Lys-Lys-Leu-Lys(CLB)-NH_2 (BP338). t_R = 7.68 and 7.83 min, corresponding to the two isomers of the 5(6)-carboxyfluorescein (97% purity). HRMS (ESI): *m/z* calcd. for $C_{110}H_{173}Cl_2N_{21}O_{19}$ $[M + 6H]^{6+}$ 360.3760; 360.3766; calcd. for $C_{110}H_{172}Cl_2N_{21}O_{19}$ $[M + 5H]^{5+}$ 432.2498; 432.2496; calcd. for $C_{110}H_{171}Cl_2N_{21}O_{19}$ $[M + 4H]^{4+}$ 540.0604; found 540.0575; calcd. for $C_{110}H_{170}Cl_2N_{21}O_{19}$ $[M + 3H]^{3+}$ 719.7447; found 719.7458.

CLB-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-Lys(CF)-NH_2 (BP339). t_R = 7.86 min (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{110}H_{173}Cl_2N_{33}O_{19}$ $[M + 6H]^{6+}$ 388.3822; found 388.3821; calcd. for $C_{110}H_{172}Cl_2N_{33}O_{19}$ $[M + 5H]^{5+}$ 465.8571; found 465.8573; calcd. for $C_{110}H_{171}Cl_2N_{33}O_{19}$ $[M + 4H]^{4+}$ 582.0696; found 582.0687; calcd. for $C_{110}H_{170}Cl_2N_{33}O_{19}$ $[M + 3H]^{3+}$ 775.7570; found 775.7576.

CF-Arg-Arg-Leu-Phe-Arg-Arg-Ile-Leu-Arg-Arg-Leu-Lys(CLB)-NH_2 (BP340). t_R = 7.47, 7.61, 7.70 and 7.79 min, corresponding to the isomers of the 5(6)-carboxyfluorescein (>99% purity). HRMS (ESI): *m/z* calcd. for $C_{110}H_{173}Cl_2N_{33}O_{19}$ $[M + 6H]^{6+}$ 388.3822; found 388.3820; $C_{110}H_{172}Cl_2N_{33}O_{19}$ $[M + 5H]^{5+}$ 465.8571; found 465.8572; $C_{110}H_{171}Cl_2N_{33}O_{19}$ $[M + 4H]^{4+}$ 582.0696; found 582.0681; $C_{110}H_{170}Cl_2N_{33}O_{19}$ $[M + 3H]^{3+}$ 775.7570; found 775.7565.

Cytotoxicity assays

Cytotoxicity of CLB and of the CLB-peptide conjugates **BP325**, **BP331**, **BP332**, **BP333**, **BP334**, **BP335**, **BP336**, **BP337** and CLB in CAPAN-1, MCF-7, PC-3 and SKMEL-28 tumor cells and in 1BR3G non-malignant cells was determined by the MTT assay. CLB-peptide conjugates were diluted in Milli-Q water to obtain 2 mM stock solutions. CLB was dissolved in DMSO to provide a 75 mM stock solution.[40] Appropriate aliquots of these solutions were diluted in the cell culture medium to obtain the final working concentrations. Aliquots of 10 000 CAPAN-1, 6000 MCF-7 or PC-3, 4000 SKMEL-28 and 5000 1BR3G cells were seeded on 96-well plates 24 h prior to the treatments. Then, cells were treated for 48 h with the corresponding compound at concentrations ranging from 0 to 100 µM. After removal of the treatment, cells were washed with PBS and incubated for additional 2 h in the dark with fresh culture medium (100 µL) with MTT (10 µL). The medium was discarded and DMSO (100 µL) was added to each well to dissolve the purple formazan crystals. Plates were agitated at room temperature for 2 min and the absorbance of each well was determined with an absorbance microplate reader (ELx800, BioTek, Winooski, USA) at a wavelength of 570 nm. Three replicates for each compound were used, and all treatments were tested at least in three independent experiments. For each treatment, the cell viability was determined as a percentage of the control untreated cells by dividing the mean absorbance of each treatment by the mean absorbance of the untreated cells. The concentration that reduces by 50% the cell viability (IC_{50}) was established for each compound using a four-parameter curve fit (Gen5 BioTeck Instruments).

Flow cytometry

The uptake efficiency of **CF-BP16**, **CF-BP308**, **BP326** (CLB-BP16-CF), **BP338** (CF-BP16-CLB), **BP339** (CLB-BP308-CF) and **BP340** (CF-BP308-CLB) by MCF-7 cells was quantified by flow cytometry. Aliquots of 50 000 cells were seeded in 24 well-plates and allowed to attach for 24 h. Next, cells were incubated with **CF-BP16** and **CF-BP308** at 25 µM for 1, 3 and 6 h or with **BP326**, **BP338, BP339** and **BP340** at 25 µM for 6 h at 37 ºC. The cells were harvested by trypsinization and washed with 2% FBS in cold PBS. The fluorescence of the cells, corresponding to the cellular uptake of the carboxyfluorescein labelled peptides, was analysed using a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with the CellQuestTM software (Becton Dickinson). The mean fluorescence intensity was represented on a four orders of magnitude log scale (1-10,000). Ten thousand cells were analysed in each experiment.

Confocal microscopy

MCF-7 cells were seeded on coverslips and allowed to attach overnight. Mitochondria were stained with 500 nM Mitotracker red for 30 min at 37 ºC. The cells were incubated with **CF-BP16** and **CF-BP308** at 50 µM for 30 min at 4 ºC and for 3 h at

37 ºC, washed with cold PBS and fixed with 4% paraformaldehyde in PBS for 15 min at 4 ºC. The cell nucleus were stained with 2 Ug ml^{-1} Hoescht 33258 (excitation/emission: 352 nm/461 nm) during 15 min at room temperature. After washing twice with cold PBS, the coverslips were mounted using a fluorescence mounting medium (Dako, Carpinteria, CA, USA) and examined using a Leica TCS-SP5 multiphoton and high-velocity spectral confocal microscope (Leica Microsystems, Nussloch, Germany).

Cathepsin B enzymatic assays

The enzymatic digestion of the CLB-peptide conjugates **BP332** (CLB-GFLG-BP16) and **BP333** (BP16-GLFG-CLB) was evaluated using a previously reported method with minor modifications.[32] A cathepsin B stock solution (1×10^4 U L^{-1}, 60 µL) was added to phosphate buffer (pH 5.0, 25 mM L-Cys, 1 mM EDTA; 890 µL) and preactivated for 10 min at 37 ºC. Next, **BP332** and **BP333** (1.8 mM, 50 µL) were added to the mixture. Aliquots of this mixture (110 µL) were sampled at 0, 10, 30, 60, 90, 120 and 180 min, flash frozen in liquid nitrogen and analyzed by HPLC-MS. The analytical HPLC conditions used for these analyses were the same as described above in the Materials and Methods section to test the purity of the peptides.

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Notes and references

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