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Structure–Activity Relationships of JMV4463, a Vectorized Cathepsin D Inhibitor with Antiproliferative Properties: The Unique Role of the AMPA-Based Vector

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Cathepsin D (CathD) is overexpressed and secreted by several solid tumors and stimulates their growth, the mechanism of which is still not understood. In this context, the pepstatin bioconjugate JMV4463 [Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(AMPA)_4$-NH_2; O_2Oc = 8-amino-3,6-dioxaoctanoyl, Sta = statine, AMPA = *ortho*-aminomethylphenylacetyl], containing a new kind of cell-penetrating vector, was previously shown to exhibit potent antiproliferative effects in vitro and to delay the onset of tumors in vivo. In this study, we performed a structure–activity relationship analysis to evaluate the significance of the inhibitor and vector moieties of JMV4463. By modifying both statine residues of pepstatin we found that the antiproliferative activity is correlated with CathD inhibition, supporting a major role of the catalytic activity of intracellular CathD in cancer cell proliferation. Replacing the vector composed of four AMPA units with other vectors was found to abolish cytotoxicity, although all of the conjugates enabled pepstatin transport into cells. In addition, the $AMPA_4$ vector must be localized at the C terminus of the bioconjugate. The unexpected importance of the vector structure and position for cytotoxic action suggests that $AMPA_4$ enables pepstatin to inhibit the proteolysis of critical CathD substrates involved in cell proliferation via a unique mechanism of action.

Introduction

Cathepsin D (CathD) is overexpressed and secreted by a number of solid tumors (breast, prostate, melanoma, colorectal cancer, glioma, etc.) and was shown to be involved in their progression and development of metastasis.[1–6] In the clinic, elevated CathD levels are related to a poor prognosis in various cancers.[7–11] At first recognized for its role in the degradation of proteins in lysosomes, CathD was also proposed to be responsible for more specific functions that control tissue regeneration[12] and that regulate essential cellular processes[13] such as the activation or regulation of various growth factors and/or the degradation of growth inhibitors,[14–17] and the inactivation of chemokines involved in the antitumor immune response.[18] CathD also contributes to the activation of anti-apoptotic proteins such as Aven and the induction of autophagy.[19–24] Therefore, CathD constitutes a pertinent target in cancer; inhibition of its intracellular or extracellular proteolytic activity could be an efficient way to help control or impede tumor growth.[15,25–29] We previously reported that a cell-penetrating CathD inhibitor (JMV4463) exhibits potent anti-proliferative effects in vitro and delays tumor emergence and growth in tests with mice xenografted with breast cancer cells.[30] Although CathD is present in all cells, the absence of observable toxicity toward normal human fibroblasts and in in vivo experiments indicates that targeting the overexpression of CathD in tumor cells can be considered as a selective strategy.

JMV4463 is composed of three main regions: 1) pepstatin, a potent CathD inhibitor ($K_D \sim 0.5$ nM),[31,32] which only poorly penetrates cells by itself; 2) a tetramer of *ortho*-aminomethylphenylacetyl residues ($AMPA_4$), which belongs to a new class of vectors based on short oligomers of constrained dipeptide mimetics called cell-penetrating non-peptides (CPNP), which target the endolysosomal pathway;[33] and 3) a hydrophilic moiety that improves aqueous solubility (Figure 1).

To determine the structural features required for efficient internalization and inhibition of tumor proliferation, we synthesized several analogues of JMV4463 (Table 1). In particular, we replaced the $AMPA_4$ vector with various cell-penetrating peptides (CPPs) and CPNPs. We also assessed the importance of the positions of the various components in the conjugate and that of the two statine residues of the pepstatin moiety.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201500457: characterization (yields, HPLC, MS data) of conjugates **1**–**19**, synthesis of Fmoc-LBD-OH **25**, and ^{1}H NMR spectra of **25** and synthetic intermediates **23** and **24**.

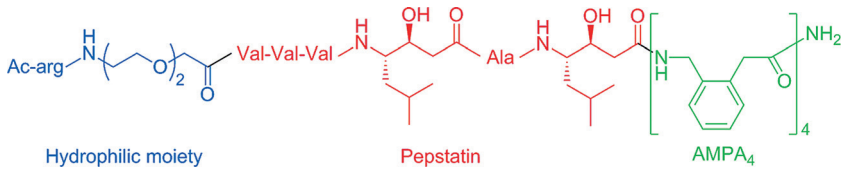

Figure 1. Structure of JMV4463, highlighting the hydrophobic, pepstatin, and $AMPA_4$ regions.

Results and Discussion

Design of bioconjugates 1–19

For solubility reasons, all bioconjugates except **12** and **13** contain a hydrophilic moiety composed of a D-arginine (arg) residue and a short poly(ethylene glycol) segment, 8-amino-3,6-dioxaoctanoyl (O_2Oc). For most of the conjugates this moiety was attached at the N terminus, but it was also placed at the C terminus in the reverse order (O_2Oc-arg, compound **9**) or partially moved (in compound **11**) (Table 1). Several analogues that differ from JMV4463 in the number of AMPA units (compounds **2**–**4** with 2, 3, and 5 units, respectively) were prepared in order to assess the optimal length required for efficient translocation and anti-proliferative activity. We also explored the significance of the position of the AMPA tetramer ($AMPA_4$) vector within the conjugate. A fully reversed conjugate ($AMPA_4$ at the N terminus, compound **9**) was prepared as well as a conjugate in which $AMPA_4$ was placed between the hydrophilic moiety and the inhibitor (compound **10**). The O_2Oc residue was moved as a spacer between pepstatin and the vector (compound **11**). Substitution of $AMPA_4$ with other vectors was also evaluated, including the highly charged and hydrophilic CPPs penetratin[34] (**12**) and octaarginine (Arg_8)[35] (**13**) as well as other uncharged CPNPs composed of D-benzothiazepine (DBT_4, compound **14**)[33] or L-benzodiazepine (LBD_4, compound **15**). Finally, we explored the significance of the *ortho*-AMPA structure by preparing the corresponding analogues with the *meta*-AMPA (compound **16**) and *para*-AMPA (compound **17**) isomers. In the case of the CPP-containing bioconjugates **12** and **13**, the hydrophilic moiety was omitted because their vector moieties (penetratin, Arg_8, respectively) are themselves highly hydrophilic.

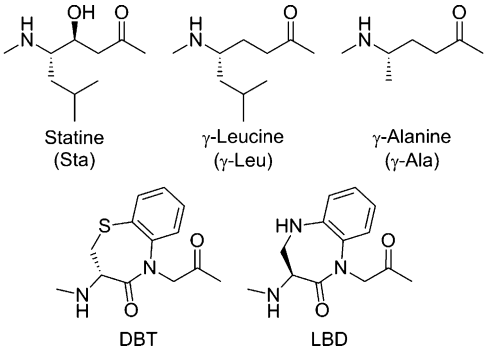

Figure 2. Structures of special residues contained in bioconjugates **1**–**19**.

The pepstatin moiety was also modified (Figure 2). The hydroxy group of the statine residue is known to be crucial for the inhibition of aspartyl proteases like CathD. To evaluate the significance of CathD inhibition in the biological activity of JMV4463, the two statine residues of the pesptatin moiety were replaced with γ-leucine (compound **5**), a modification reported to lead to a less potent inhibitor,[36] or with γ-alanine residues (compound **6**), which should further decrease the inhibitory activity. Such compounds should confirm the correlation between antiproliferative activity and CathD inhibition. The importance of each statine residue was also evaluated by replacing them with a γ-alanine residue (**7** and **8**). Finally, negative controls were included, which were previously shown to be non-cytotoxic.[30] Compound **18** is composed of the hydrophilic and pepstatin moieties, but is unable to efficiently penetrate cells. Compound **19** is composed of the hydrophilic and $AMPA_4$ moieties and does not inhibit CathD.

Table 1. Structures of bioconjugates **1**–**19**.

Compd	Structure	Compd	Structure
1[a]	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(AMPA)_4$-NH_2	**11**	Ac-arg-$(Val)_3$-Sta-Ala-Sta-O_2Oc-$(AMPA)_4$-NH_2
2	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(AMPA)_2$-NH_2	**12**	Iva-$(Val)_2$-Sta-Ala-Sta-penetratin-NH_2
3	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(AMPA)_3$-NH_2	**13**	Iva-$(Val)_2$-Sta-Ala-Sta-$(Arg)_8$-NH_2
4	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(AMPA)_5$-NH_2	**14**	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(DBT)_4$-NH_2
5	Ac-arg-O_2Oc-$(Val)_3$-**γLeu**-Ala-**γLeu**-$(AMPA)_4$-NH_2	**15**	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-$(LBD)_4$-NH_2
6	Ac-arg-O_2Oc-$(Val)_3$-**γAla**-Ala-**γAla**-$(AMPA)_4$-NH_2	**16**	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-(*m*AMPA)$_4$-NH_2
7	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-**γAla**-$(AMPA)_4$-NH_2	**17**	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-(*p*AMPA)$_4$-NH_2
8	Ac-arg-O_2Oc-$(Val)_3$-**γAla**-Ala-Sta-$(AMPA)_4$-NH_2	**18**	Ac-arg-O_2Oc-$(Val)_3$-Sta-Ala-Sta-NH_2
9	Ac-$(AMPA)_4$-$(Val)_3$-Sta-Ala-Sta-O_2Oc-arg-NH_2	**19**	Ac-arg-O_2Oc-$(AMPA)_4$-NH_2
10	Ac-arg-O_2Oc-$(AMPA)_4$-$(Val)_3$-Sta-Ala-Sta-NH_2		

[a] Bioconjugate **1** = JMV4463.

All pepstatin conjugates containing a CPNP vector should be stable toward CathD, and no cleavage of either the vector or the hydrophilic moiety is expected before interaction of pepstatin with CathD. Indeed, the amide links between pepstatin and each surrounding moiety involve at least one non-α-amino acid residue. These conjugates should also be highly stable in cells toward any peptidase, although the Val_3 segment of pepstatin moiety could be considered as a potential cleavage site. All compounds were synthesized by standard solid-phase synthesis following the Fmoc strategy and using HBTU/DIEA for coupling, as previously described.[30]

Inhibitory potency against purified CathD

We first assessed the inhibitory potency of the bioconjugates against purified CathD (Table 2). All compounds containing unmodified pepstatin (**1**–**4**, **9**–**18**) showed inhibitory activity similar to that of pepstatin A, as they exhibited comparable inhibition percentages at 10 μM (>90%) and 10 nM (>85%), indicating that the presence of the vectors and the hydrophilic moiety do not interfere with pepstatin recognition by the enzyme. As hypothesized, compound **5** containing two γ-leucine residues in place of the two statines showed a lower inhibitory activity (~63% at 10 μM), while compound **6**, with two γ-alanine residues, exhibited almost no inhibition at the same concentration. Replacing only one statine residue by γ-alanine led to compounds **7** and **8** with inhibitory potencies similar to that of pepstatin A when tested at 10 μM. However, only compound **7** retained high inhibitory potency at 10 nM, similar to all pesptatin-containing compounds. These results indicate the important role of the first statine residue in the sequence of pepstatin for potent CathD inhibition. They are in agreement with previously published data concerning pepstatin A.[37] Finally, IC_{50} values were determined for representative compounds (**1**, **5**–**7**, **9**, and **18**) and were found to correlate with the values at 10 μM and 10 nM.

Table 2. Inhibition of proteolytic CathD activity by bioconjugates **1**–**19**.[a]

Compd	IC_{50} [nM][b]	Purified CathD Inhib. [%][b]		Cell CathD Inhib. [%][c]
		10 μM	10 nM	
1	2.30±0.09	95±2*	93±1*	94±4*
2	–	89±7*	89±1*	9±19
3	–	84±5*	90±1*	2±9
4	–	81±3*	86±7*	85±8*
5	41.00±1.76	63±2*	22±2	28±11
6	>10000	13±5	7±4	1±9
7	4.40±0.10	94±2*	91±2*	91±4*
8	–	92±1*	15±8	96±4*
9	1.10±0.03	91±1*	89±1*	97±2*
10	–	94±5*	81±7*	8±8
11	–	91±1*	88±1*	95±3*
12	–	90±2*	85±4*	91±7*
13	–	93±2*	80±2*	85±2*
14	–	92±2*	88±1*	98±1*
15	–	94±1*	90±1*	78±2*
16	–	92±1*	90±1*	98±3*
17	–	92±1*	86±1*	93±4*
18	2.80±0.16	92±3*	87±1*	8±14
19	–	1±6	1±4	6±13
pepstatin A[d]	–	95±1*	–	10±10*
DMSO	–	0±9	0±8	0±5

[a] The enzymatic activity of CathD was determined by FRET using a peptide substrate coupled to a fluorophore (EDANS) and a quencher (DABCYL), measured at λ 355 nm in a microtiter plate reader after 90 min incubation at 37 °C with 5 μM substrate at pH 3.5. Percent CathD inhibition is reported for bioconjugates **1**–**17** together with **18**, **19**, and 1% DMSO as negative controls. [b] Inhibition of CathD activity determined by incubation of isolated CathD (250 ng) with test compounds at either 10 μM or 10 nM final concentration in cell-free conditions; a 50–1 nM concentration range was used for IC_{50} determination. [c] Inhibition of intracellular CathD: after incubation of MDA-MB-231 cells for 24 h with test compounds at 10 μM, cellular CathD activity was determined in 50 μg cellular proteins. Values are the mean±SD of three separate experiments; $^*p<0.05$ relative to DMSO control (Student *t*-test). [d] Data from Ref. [30].

Capacity to penetrate cells: measurement of intracellular CathD activity

The ability of the compounds to penetrate cells was assessed with MDA-MB-231 tumor cells. After 24 h incubation at a concentration of 10 μM, the cells were washed, lysed, and supernatants were acidified and submitted to enzymatic tests to measure the levels of CathD inhibition (Table 2). The reported inhibition is only related to the ability of conjugates to penetrate cells, but does not indicate in which cell compartment they are localized. When comparing compounds **1**–**4**, which differ in the number of AMPA units present, only compounds **1** and **4** were found to potently inhibit CathD proteolytic activity. This indicates that a minimum of four AMPA units is required to enable cell penetration. As expected, compound **5**, with two γ-leucine residues, showed low intracellular CathD inhibitory activity (~28%) in accordance with its low activity against isolated CathD, whereas compound **6**, with γ-alanine residues was found to be totally inactive. Compounds **7** and **8**, containing only one statine residue, were equally potent to **1** in inhibiting intracellular CathD activity. It is important to note that compound **8** showed >90% inhibition on living cells, although it was less potent than compounds **1** and **7** toward purified CathD at 10 nM. This suggests that the bioconjugate efficiently penetrates, allowing sufficiently high intracellular concentrations to be reached.

Overall, it is notable that the efficiency of $AMPA_4$ to deliver pepstatin was extremely high, as it allows a near total inhibition of the elevated CathD concentration (≥50 pmol mg^{-1} cellular proteins) present in these cancer cells. Compounds **9**–**11**, which differ from **1** by the order of their functional moieties (hydrophilic moiety, pepstatin, $AMPA_4$) gave disparate results. The presence of the vector at the N terminus (**9**) or at the C terminus (**11**) induced intracellular CathD inhibition similar to that of **1**. In the case of compound **11**, this result shows that it is possible to introduce a spacer between the vector and the enzyme inhibitor while maintaining intracellular delivery. However, insertion of the vector between the hydrophilic moiety and pepstatin (in **10**) abolished the activity, suggesting the inability of this bioconjugate to penetrate cells. We have shown that JMV4463 (**1**) penetrates cells via the endolysosomal pathway,[30] but its mode of interaction with membranes and of in-

ternalization has not yet been determined. It can be hypothesized that the highly hydrophobic $AMPA_4$ vector requires location at one end of the bioconjugate for efficient internalization, whereas the presence of the two neighboring moieties on both sides in compound **10** would prevent cell membrane interaction.

Finally, the ability of other vectors to translocate pepstatin into cells was evaluated. As expected, the CPPs penetratin (**12**) and polyarginine Arg_8 (**13**) enabled pepstatin to enter cells as indicated by the strong inhibition of intracellular CathD measured after cell lysis. In addition, the two CPNP vectors DBT_4 (**14**) and LBD_4 (**15**) also led to intracellular inhibition. The latter was found to be slightly less efficient (~78% inhibition for **15** instead of >94% for compounds **1** and **14**), suggesting that replacing the sulfur atom (DBT) with an NH group (LBD) led to lower penetration efficiency. Strikingly, bioconjugates **16** and **17** were found to be as efficient as compound **1** in inhibiting intracellular CathD, indicating that the *meta*- and *para*-AMPA tetramers (*m*$AMPA_4$ and *p*$AMPA_4$, respectively) are able to translocate pepstatin into cells. Interestingly, the vectors LBD_4, *m*$AMPA_4$ and *p*$AMPA_4$ have never been reported before, and the present results suggest that the family of CPNPs can be further broadened.

Toxicity toward cancer cells

The cellular toxicity of analogues at 10 μM was evaluated with MDA-MB-231 breast cancer cells after an incubation period of three days (Figure 3). As expected, bioconjugates **2**, **3**, **6**, **10** and the negative controls **18** and **19**, which were unable to inhibit intracellular CathD, were found to be non-cytotoxic. Consistent with its intermediate CathD inhibitory activity, compound **5** showed intermediate cytotoxicity. Therefore, the results obtained for compounds **1**, **5** (two γ-leucine residues), and **6** (two γ-alanine residues) showed that the antiproliferative effect of compound **1** (JMV4463) is related to intracellular CathD inhibition. For all other inhibitor conjugates shown to enter cells (Table 2), the results highlight that their internalization does not necessarily lead to cytotoxicity.

Conjugates containing unmodified pepstatin or pepstatin with only one statine residue together with an $AMPA_n$ ($n=4,5$) vector located at their C terminus (**4**, **7**, **8**, and **11**) exhibited cytotoxicity similar to that of JMV4463 (compound **1**). It is interesting to note that it was possible to introduce a spacer between the CathD inhibitor and the vector (compound **11**) without loss of activity and that only one statine residue (compounds **7** and **8**) was sufficient to induce cell toxicity. The latter result was further confirmed by comparing the activities of compounds **7** and **8** with that of **1** in a concentration–response experiment, which showed a very good superimposition of the three cytotoxicity curves (Figure 4).

However, changing the AMPA-based vector to a CPP (**12**, **13**) or a different CPNP (**14**, **15**) fully abolished cytotoxicity. More strikingly, isomers **16** and **17** were found to be non-cytotoxic, showing that the *ortho* arrangement of the AMPA aminomethyl and acetyl groups is crucial. These results might be ex-

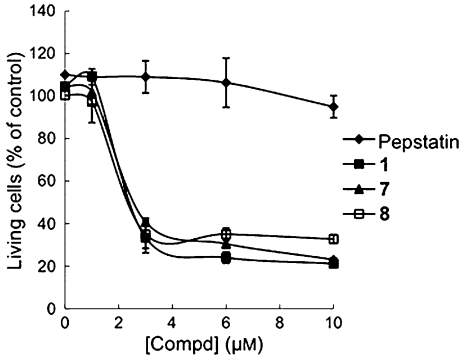

Figure 4. Viability of MDA-MB-231 breast cancer cells. Cells were treated with pepstatin (control) and with compounds **1**, **7**, and **8** for three days, all at 1, 3, 6, and 10 μM. Growth was evaluated by MTT assay as described in the Experimental Section. Values are expressed as a percentage of control cells treated with vehicle alone (1% DMSO) and are the mean ± SD of three independent experiments.

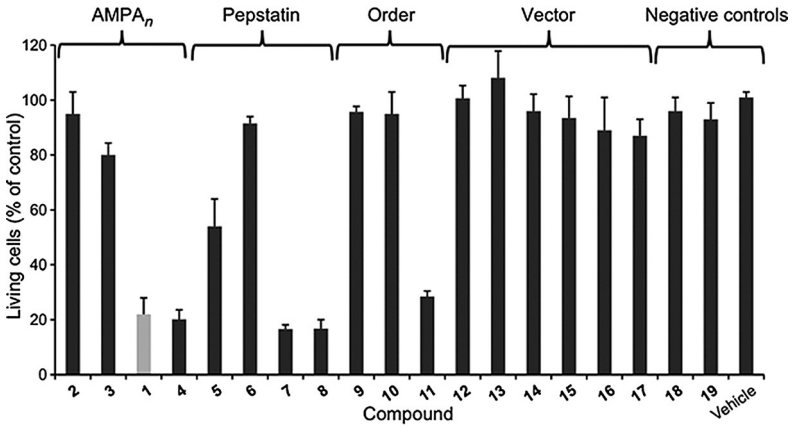

Figure 3. Cytotoxicity of compounds **1**–**19** toward MDA-MB-231 breast cancer cells. Cells were treated with each compound at 10 μM for three days, and growth was evaluated by MTT assay as described in the Experimental Section. All values are expressed as a percentage of control cells treated with vehicle alone (1% DMSO) and are the mean ± SD of three independent experiments. The different types of structural variations are indicated at top ($AMPA_n$: variable AMPA oligomer length, Pepstatin: substitution of statine residues, Order: order of the three functional moieties, Vector: vectors other than $AMPA_4$).

plained by an insufficient addressing of these conjugates to the CathD-containing compartment and/or to a rapid externalization from endosomes following endocytosis. In the case of the DBT_4-containing bioconjugate **14**, its inactivity on cell growth was unexpected, as the DBT vector was shown to very efficiently penetrate cells via the endolysosomal pathway.[33] The reasons for this behavior are unclear, but it is clear that $AMPA_4$ possesses specific properties not shared by other vectors. Compound **9**, which presents a reverse composition relative to **1**, was shown to penetrate cells (Table 2), but is not cytotoxic. This result indicates that the position of the $AMPA_4$ vector is crucial, as already highlighted by the results obtained with the non-cell-penetrating compound **10**. Altogether, these results suggest that a restrictive and specific mode of interaction between CathD and its inhibitor occurs inside the cell compartment, which could be achieved only by bioconjugates containing the $AMPA_4$ vector at the C terminus.

Conclusions

This structure–activity study points to a major role of the catalytic activity of intracellular CathD in the proliferation of breast cancer cells and to the critical importance of the $AMPA_4$ vector. In addition, we found it possible to remove one of the two statine residues of the pepstatin moiety, allowing a more economical chemical synthesis.

Concerning the pepstatin moiety of the conjugate, its relevance in antiproliferative activity is clearly evidenced by the loss of activity resulting from substitution of both statine residues with non-hydroxylated γ-amino acids. This lends significant support to previous studies, indicating that the mitogenic activity of CathD is at least partially due to its catalytic activity either by activation of growth factor precursors or by inactivation of growth inhibitors.[14–16] JMV4463 is an inhibitor of CathD catalytic activity with high clinical potential and is also a highly valuable tool to explore the mechanisms of cancer cell proliferation involving intracellular CathD.

The translocation of pepstatin bioconjugates into cells was enabled by all selected vectors, but their antiproliferative activity was strictly dependent on the structure of the vector and its position. It is clear that $AMPA_4$ possesses a unique mode of action, the mechanism of which is not yet understood. This finding suggests that the structure of $AMPA_4$ imparts the bioconjugate with the capacity to prevent the proteolysis of critical substrates involved in cell proliferation. CathD is considered a multifunctional enzyme, and its precursor pro-CathD undergoes several steps of maturation during cell trafficking, and several binding partners have also been identified.[13] CathD could perform various functions during the cell trafficking. In addition, active but not fully matured forms of CathD, such as the 48 kDa form, are present in endosomes and they probably contribute to these functions. We therefore hypothesize that thanks to the $AMPA_4$ vector, JMV4463 can more efficiently recognize immature forms of CathD and/or localize more efficiently in the CathD-containing compartment involved in the observed effect. These hypotheses are now under investigation to understand the specific role of the vector.

Experimental Section

Synthesis of compounds 1–19

Fmoc-protected amino acid derivatives, HBTU and Fmoc Rink amide polystyrene resin (100–200 mesh, 0.46 mmol g^{-1}) were purchased from Iris Biotech. Fmoc-(3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid (Fmoc-statine), Fmoc-γLeu-OH and Fmoc-γAla-OH were purchased from NeoMPS (Strasbourg, France). Other reagents and solvents were purchased from Riedel-de-Haën, Carlo Erba or Acros organics and used without purification. Solvents used for HPLC and LC–MS were of HPLC grade. All final compounds were purified by reversed-phase HPLC, and the purity assessed by analytical reversed-phase HPLC was found to be $>95\%$. Fmoc-AMPA-OH and Fmoc-DBT-OH were prepared as previously described.[38,39] The synthesis of Fmoc-LBD-OH (compound **25**) is described in the Supporting Information.

Anchoring on Rink amide PS resin. Fmoc-Rink amide resin was conditioned for 30 min in DMF and submitted to the standard deprotection cycle, using DMF/piperidine (80:20 *v*/*v*) solution for 30 min. After washing steps, the first residue was loaded onto the resin through a standard coupling cycle.

Coupling. The coupling reaction was carried out manually in plastic syringes equipped with frits. HBTU (3 equiv) as coupling agent, DIEA (3 equiv) as base, and Fmoc-AA-OH (3 equiv) (according to resin loading) were dissolved in DMF and added to the resin. The reaction was stirred for 3 h at room temperature. The reaction was monitored by the standard TNBS test.[40]

Deprotection. Fmoc group removal of Fmoc-protected amino acids, except Fmoc-AMPA-OH, was carried out using DMF/piperidine (80:20 *v*/*v*) solution twice for 30 min. Fmoc group removal of Fmoc-AMPA-OH was carried out using a solution of DMF/piperidine/DBU (92:4:4 *v*/*v*/*v*) twice for 30 min.

Washing steps were performed after each coupling and deprotection step. Once with MeOH, once with CH_2Cl_2, three times with DMF, and three times again with CH_2Cl_2.

Cleavage of Rink amide PS resin. Bioconjugates were simultaneously cleaved from resin and deprotected for 2 h in a mixture of TFA, TIS, and H_2O (95:2.5:2.5 *v*/*v*/*v*). After removal of the resin by filtration, the TFA was concentrated in vacuo. Compounds were precipitated by the addition of Et_2O and filtered. They were dissolved in a solution of CH_3CN/H_2O (50:50 *v*/*v*) containing 0.1% TFA and freeze dried.

Purification. All bioconjugates were purified by preparative HPLC (Waters 4000 apparatus) carried out on a C_{18} reversed-phase column (C_{18} Deltapak column, 100 mm × 40 mm, 15 μm, 100 Å) with a mixture of $H_2O + 0.1\%$ TFA and $CH_3CN + 0.1\%$ TFA in gradient mode at a flow rate of 50 mL min^{-1} with UV detection at λ 214 nm. HPLC and MS analytical data of the bioconjugates **1**–**19** are given in Supporting Information Table 1.

Biological evaluation

Cell culture and viability assay. Human breast MDA-MB-231 cancer cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and were allowed to grow in a humidified atmosphere at 37 °C under 5% CO_2. MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cell viability was assessed in 96-well plates by the MTT method after treatment of ad-

herent cells with compounds or with 1% DMSO (vehicle) for three days. A solution of MTT (0.5 mg mL^{-1}) was added to each well and incubated for 4 h. The MTT-formazan product was dissolved in EtOH/DMSO (50:50) and estimated by measuring absorbance at λ 540 nm in a Multiskan FC plate reader (Fisher Scientific, Illkirch, France).

CathD proteolytic activity. To detect CathD protease activity, Förster resonance energy transfer (FRET) was used. A fluorophore (EDANS) and a quencher (DABCYL) dye were coupled to the C- and N-terminal ends of a peptide substrate highly selective for CathD. On intact peptides, the emission energy of the fluorophore was captured by the quencher. Following cleavage of the substrate, the quencher is no longer able to absorb the fluorescent energy of the fluorophore, and this increase in fluorescence was measured. The FRET substrate for CathD (JMV3672, DABCYL-βAla-Arg-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Asp(EDANS)-NH_2) was not commercially available and was synthesized according to standard procedures. The peptide sequence used was reported to be cleaved by CathD, but not by other cathepsins.[41] In the first case, purified recombinant human CathD (250 ng) was incubated in 0.1 M citrate buffer (pH 3.5) with 10 μM and 10 nM compounds as described.[41] In the second case, the compounds were incubated for 24 h with living cells, then cells were washed with phosphate-buffered saline (PBS), lysed and supernatants used for FRET analysis. The substrate (final concentration 5 μM) was then incubated at 37 °C with 20 μL of cell lysate supernatants at pH 3.5. After 90 min, the plate was read in a PerkinElmer 1420 Victor 2 fluorescent plate reader at λ_{ex} = 355 nm and λ_{em} = 538 nm. The background fluorescence of the FRET substrate was later subtracted out. Results were presented as percent of inhibition of CathD activity.

Abbreviations. AMPA: *ortho*-aminomethylphenylacetyl, CathD: cathepsin D, CPNP: cell-penetrating non-peptide, CPP: cell-penetrating peptide, DABCYL: 4-([4-(dimethylamino)phenyl]azo)benzoyl, DBT: D-benzothiazepine, DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene, DIEA: *N*,*N*-diisopropylethylamine, DMF: *N*,*N*-dimethylformamide, DMSO: dimethyl sulfoxide, EDANS: 5-([2-aminoethyl]amino)naphthalene-1-sulfonyl, Fmoc: 9-fluorenylmethyloxycarbonyl, HBTU: *N*-[(1*H*-benzotriazol-1-yloxy)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate, LBD: L-benzodiazepine, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, O_2Oc: 8-amino-3,6-dioxaoctanoyl, PS: polystyrene, TFA: trifluoroacetic acid, TIS: triisopropylsilane, TNBS: trinitrobenzenesulfonic acid.

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Keywords: antiproliferation · cathepsin D · cell-penetrating non-peptides · pepstatin delivery · structure–activity relationships

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