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Coupling of the Antennapedia Third Helix to a Potent Antagonist of the p53/hdm2 Protein–Protein Interaction

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Abstract—The use of cell-membrane translocating sequences for intracellular delivery of peptides can be a powerful approach to validate drug discovery targets in cellular settings. To accomplish this, a protocol has been implemented to couple the antennapedia third helix (residues 43–58) to a potent antagonist of the p53/hdm2 protein–protein interaction without affecting its in vitro inhibitory activity.

Introduction

The tumor suppressor activity of p53 is negatively regulated by the human double minute 2 (hdm2) protein.[1] Upon binding to the N-terminus of p53,[2] hdm2 inhibits the transcriptional activity of p53 and induces its nuclear export into the cytoplasm, where p53 is degraded by the ubiquitin pathway.[3] Inhibition of this protein–protein interaction in tumor cells expressing the wild-type p53 protein and having a functional p53 pathway should induce tumor cell death by apoptosis. The potential application of this approach for cancer treatment has prompted us[4] and others[5] to initiate drug discovery programs to identify inhibitors of the p53/hdm2 protein–protein interaction. Recently, we have reported the discovery of Ac-Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3c-Leu-NH_2 (peptide **1**, Table 1), which is a highly potent in vitro peptide antagonist of the interaction between hdm2 and the tumor suppressor p53.[6] In cell-based assays, this peptide induces the accumulation of p53 and $p21^{Waf1/Cip1}$ in tumor cells albeit at high concentrations.[7] To improve its cellular permeability and expand its use as a tool compound to study the activation of the p53 pathway in tumor cells, we have developed a protocol for coupling the antennapedia third helix (Arg^{43}-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys^{58})[8] to peptide **1** without affecting its original in vitro p53/hdm2 inhibitory activity.

Chemistry and Results

The synthesis of peptides **1** and **2** has been described in previous publications from our group.[6,7] The octapeptides (peptides **6** and **7**, Fig. 1) were synthesized manually starting with a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin[9] for establishing the required C-terminal carboxamide and using standard solid-phase protocols based on fluorenylmethoxycarbonyl (Fmoc) chemistry.[10] 4-Maleimidobutyric acid (2.0 equiv) was incorporated with *O*-(1,2-dihydro-2-oxo-1-pyridyl)-1,1,3,3-tetramethyluronium tetrafluoroborate[11] (2.0 equiv, first coupling) and *N*-[(dimethylamino)1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylmethanaminium hexafluorophosphate *N*-oxide[12] (2.0 equiv, second coupling) in the presence of diisopropylethylamine (2.2 and 6.0 equiv, respectively). The antennapedia third helix peptide (peptide **5**, Fig. 1)[8] was synthesized on a Milligen 9050 peptide synthesizer (continuous flow), employing protocols previously reported from our laboratory.[13] Biotin was incorporated using the coupling agents mentioned above for 4-maleimidobutyric acid. The dipeptide Ser-Gly was used as a spacer between the N-terminal probe and the antennapedia third helix. The completed peptide resins were simultaneously deprotected and cleaved by treatment with trifluoroacetic acid/water/1,2-ethanedithiol (76:4:20, v/v/v; resin **5**) or trifluoroacetic acid-water (19:1, v/v; resins **6** and **7**) for 3 h at room temperature. The filtrate from each cleavage reaction was added to diisopropyl ether/petroleum ether (1:1, v/v) at 0 °C, and the resulting precipitate was collected by filtration. The crude peptides were purified by medium-pressure liquid chromatography (MPLC) on a C_{18}-column using an acetonitrile–water gradient. Racemic

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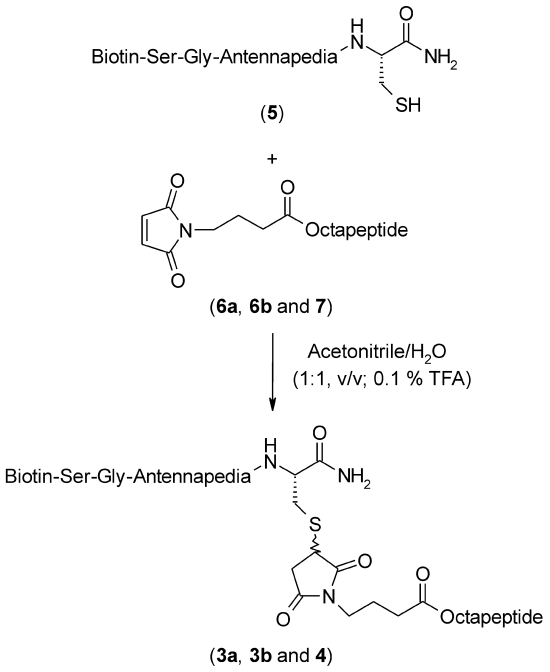
Table 1. Inhibitory potential of hdm2-binding peptides in a p53/hdm2 (GDT-hdm2$^{1-188}$) competition assay[a]

Entry	Peptide sequence	IC_{50} (nM)
1	Ac-Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3c-Leu-NH_2[b]	5 ± 1
2	Ac-Phe-Met-Aib-Pmp-Ala-Glu-Ac_3c-Leu-NH_2	>2000
3b	Biotin-Ser-Gly-Antennapedia$^{43-58}$-Cys-*linker*-Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3c-Leu-NH_2[c]	9 ± 2
4	Biotin-Ser-Gly-Antennapedia$^{43-58}$-Cys-*linker*-Phe-Met-Aib-Pmp-Ala-Glu-Ac_3c-Leu-NH_2	$>50{,}000$

[a]IC_{50} concentration to inhibit the binding of human wild-type p53 to GST-hdm2$^{1-188}$. The errors quoted correspond to the standard error in the fit of the data.
[b]Single isomer. The synthesis of this peptide is described in ref 6.
[c]The activity reported for this peptide corresponds to the one observed for the most active epimer. On the basis of the activity observed for previous compounds and our computational studies,[6] we can assume that the configuration of the 6-chloro-tryptophan in the most active epimer (**3b**) is L.



Antennapedia:

Arg^{43}-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys^{58}

Octapeptides:

Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3C-Leu-NH_2 **6a**

Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3C-Leu-NH_2 **6b**

Phe-Met-Aib-Pmp-Ala-Glu-Ac_3C-Leu-NH_2 **7**

Figure 1. Coupling of the antennapedia third helix (residues 43–58) to the octapeptides containing the N-terminal maleimido moiety. Racemic N$^{\alpha}$-Fmoc-6-chloro-tryptophan was used in the solid-phase synthesis of peptide **6** and the two epimers (**6a** and **6b**) were separated to homogeneity by MPLC.

N$^{\alpha}$-Fmoc-6-chloro-tryptophan was used in the synthesis of peptide **6** and the two epimers (**6a** and **6b**) were separated to homogeneity by MPLC.[14] Coupling of the biotin labeled antennapedia peptide (1.0 equiv; peptide **5**) to the octapeptides containing the N-terminal maleimido moiety (1.2 equiv; peptides **6a**, **6b** and **7**) was performed under nitrogen atmosphere in a previously degassed water/acetonitrile (1:1, v/v; 0.1% trifluoroacetic acid) solution. The reaction was monitored by analytical HPLC. After disappearance of peptide **5** (3 days), the crude peptides (**3a**, **3b** and **4**) were purified by MPLC on a C_{18}-column using an acetonitrile–water gradient. The purity of the final compounds was verified by reversed-phase analytical HPLC and the identity was assessed by correct mass spectral and amino acids analyses.[15]

The inhibition of the p53/hdm2 protein–protein interaction by the synthetic peptides (Table 1) was determined in an in vitro assay previously described by our group.[4a] In this competition assay, the ELISA plates were coated with GST-hdm2$^{1-188}$. After adding the peptide and full-length wild-type p53, the fraction of p53 bound to hdm2 was detected with a series of antibodies. Peptide **1** and **3b** showed similar inhibitory values in the p53/hdm2 competition assay (Table 1), confirming that the antennapedia third helix does not impair the in vitro biological activity of the parent antagonist. The lack of activity observed for peptide **4** correlates with the poor activity observed for peptide **2** (Table 1), and shows that the hybrid peptide is inactive if the parent antagonist has no biological activity.

Discussion

In our drug discovery activities in oncology, we are interested in blocking the interaction of hdm2 with the tumor suppressor p53. The disruption of this protein–protein interaction in tumor cells containing wild-type p53 and a functional p53 pathway should lead to accumulation of p53 and activation of its tumor suppressor function. Using a structure-based design approach, we have been able to identify highly potent peptide inhibitors of the p53/hdm2 protein–protein interaction.[6] These peptides have been valuable tool compounds to study the activation of the p53 pathway in tumor cells,[7] but their low cellular permeability has been an important limitation in their use in some cellular settings. To overcome this hurdle, we decided to derivatize our most potent inhibitor (peptide **1**, Table 1) and a negative control compound (peptide **2**, Table 1) with the antennapedia third helix, which is a cell-membrane translocating sequence used for the intracellular delivery of oligonucleotides and oligopeptides.[8] In our first approach, the octapeptides were synthesized in tandem with the antennapedia third helix (e.g., Biotin-Ser-Gly-Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3c-Leu-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2).[16] This straightforward procedure was hampered

by the problems faced during the solid-phase synthesis of the tandem peptides and the purification and separation of the two epimers.[17] As an alternative to this synthetic protocol, the octapeptides and the antennapedia third helix were synthesized separately and coupled through a maleimido group. To this end, the octapeptides were prepared with an additional N-terminal maleimido group (peptides **6a**, **6b** and **7**; Fig. 1) and the antennapedia third helix was appended at the C-terminus with a cysteine residue (peptide **5**; Fig. 1). The 4-maleimidobutyric acid was selected to provide the attachment point for the antennapedia third helix and to act as a spacer between the cell-membrane translocating sequence and the antagonist in order to minimize undesired interactions between them. The coupling between the antennapedia third helix and the octapeptides was carried out as described above, and the target peptides were obtained in good yields and high purity after medium-pressure liquid chromatography.

The effect of the antennapedia third helix on the biological activity of the original octapeptides was determined by testing the hybrid peptides in our in vitro p53/hdm2 competition assay.[4a] As shown in Table 1, peptides **1** and **3b** showed very similar inhibitory activities ($IC_{50} = 5$ nM vs $IC_{50} = 9$ nM). This result confirms that no undesired interactions exist between the vector (antennapedia third helix) and the cargo (octapeptide). Furthermore, it shows that the vector does not create additional intramolecular interactions or steric clashes with hdm2 that affect the biological activity of the peptide. This conclusion is further supported by the lack of activity observed for peptide **4** ($IC_{50} > 50$ μM, Table 1), which is a hybrid peptide that carries a poorly active peptide (peptide **2**, $IC_{50} > 2$ μM, Table 1). Currently, the hybrid peptides **3b** and **4** are under evaluation to study the activation of the p53 pathway in different tumor cell lines (e.g., HCT-116 and OSA-CL).[18] Peptide **4** is used as a negative control in these cell-based studies.

Protein–protein interactions are a ubiquitous mode of transmitting extracellular and intracellular signals.[19] Despite the importance of this mechanism for signal transduction, the investigation of intracellular events controlled or triggered by these interactions has been somewhat compromised by the paucity of compounds that are effective in cell-based assays. The synthetic protocol reported in this letter expands the available approaches for coupling a cell-membrane translocating sequence and a peptide. The intracellular delivery of exogenous compounds (e.g., peptides or oligonucleotides) by this system can be a powerful strategy to validate in cellular settings drug discovery targets before substantial investments are made in medicinal chemistry.

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14. The purity of the peptides was verified by reversed-phase analytical HPLC on a Chromolith SpeedROD RP18e column (50×4.6 mm): linear gradient over 2.5 min of MeCN/0.09% TFA and H_2O/0.1% TFA from 1:49 to 3:2 (gradient 1) or 1:49 to 1:0 (gradient 2); flow rate 4.0 mL/min, detection at 215 nm; single peak at $t_R = 1.99$ (**5**; gradient 1) min $t_R = 2.00$ min (**6a**; gradient 2); $t_R = 2.04$ min (**6b**; gradient 2); and $t_R = 1.82$ min (**7**; gradient 2). Mass spectral analyses (matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, MALDI-TOF) revealed molecular masses within 0.1% of the expected values (positive- or negative-ion mode): 2720.0 (calcd 2720.4; peptide **5**); 1332.5 (calcd 1331.9; peptide **6a**); 1332.4 (calcd 1331.9; peptide **6b**) and 1182.6 (calcd 1182.3; peptide **7**).

15. The purity of the peptides was verified as described in ref 14. Analytical HPLC: single peak at $t_R = 1.76$ min (**3a**; gradient 2); $t_R = 1.77$ min (**3b**; gradient 2); and $t_R = 1.69$ min (**4**; gradient 2). Mass spectral analyses (MALDI-TOF; negative-ion mode): 4051.6 (calcd 4051.3; peptide **3a**); 4051.1 (calcd 4051.3; peptide **3b**) and 3901.2 (calcd 3901.7; peptide **4**). Quantitative amino acid analyses of the peptides revealed the expected amino acid compositions.
16. Biotin was incorporated at the N-terminus of the peptide to provide the probe that can be recognized by a labeled detection reagent (e.g., fluorescent streptavidin).
17. In spite of our purification efforts, we were unable to separate the two epimers of Biotin-Ser-Gly-Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3c-Leu-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2. The purified peptide (mixture of the two epimers) showed an IC_{50} value of 96 ± 4 nM in our in vitro p53/hdm2 competition assay.
18. Initial experiments have been performed using OSA-CL tumor cells in a cell proliferation assay (see ref 7 for experimental details on this assay). OSA-CL is a cell line that overexpresses the hdm2 protein and contains wild-type p53 (see: Bottger, A.; Botther, V.; Sparks, A.; Liu, W. L.; Howard, S. F.; Lane, D. P. *Curr. Biol.* **1997**, *7*, 860). Peptide **3b** causes 30% growth inhibition of OSA-CL cells at $c = 50$ nM. The interpretation of this result is hampered by the high toxicity observed in these cells with the Biotin-Ser-Gly-Antennapedia$^{43-58}$ peptide alone. The identification of a more suitable tumor cell line is currently under investigation.
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