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AApeptides as a New Class of Peptidomimetics to Regulate Protein-Protein Interactions

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1. Introduction

In human physiology, proteins at all times are synthesized, processed, degraded and post-translationally modified to varying degrees and at different rates, for their participations in a wide variety of activities to maintain normal functions of the body (Murray et al. 2007). In the successful proceedings of all the biological events, signals are consistently received and sent via physical contacts between proteins (Murray et al. 2007). The communication between proteins or the alternatively called non-covalent protein-protein interactions are thereby considered as important as proteins' own functions (Murray et al. 2007). Disruptions of these signaling pathways by either mutational changes or deregulation of one of the protein partners would result in a series of diseases (Murray et al. 2007). On the other hand, therapeutic approaches based on chemical agents could potentially inhibit protein-protein interactions, thereby restoring the balance of signaling pathways, and leading to the cure of diseases (Murray et al. 2007). However, it is quite challenging to develop chemical agents which can target protein-protein interactions (Arkin et al. 2004; Whitty et al. 2006). Unlike the traditional medicinal chemistry approach, in which small molecule inhibitors are developed to target the hydrophobic pocket of enzymes / kinases, chemical agents are now required to bind to large surfaces of proteins that are usually amphiphilic and flexible (Murray et al. 2007). Yet a number of successful stories have been reported (Murray et al. 2007). Taking the p53/MDM2 system as an example, the p53/MDM2 has been a model system for the inhibition of protein-protein interactions, and has been reported to be the targets of a wide variety of inhibitors (Oren 1999; Balint et al. 2001; McLure et al. 2004; Brooks et al. 2006).

The tumor suppressor protein p53 is a transcription factor that executes multiple anticancer functions. Through its binding to DNA, p53 can initiate the expression of several important proteins, which are responsible for DNA repair, induction of growth arrest to hold the cell

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cycle at the G1/S regulation point, as well as the initiation of apoptosis (Lowe et al. 1993; Pellegata et al. 1996; Liu et al. 2001). However, at the normal state, the p53 activity is down-regulated by the murine double minute 2 protein (MDM2) which binds to the α-helical transactivation domain near the N-terminus of p53 (Momand et al. 1992; Oliner et al. 1992). Cocrystal structure studies revealed that three hydrophobic side chains from Phe19, Trp23, and Leu26 of p53 make direct contacts with MDM2 and account for the primary interactions (Kussie et al. 1996). The binding of MDM2 not only inhibits p53 DNA-binding activity but also induces the proteosomal degradation of p53 (Haupt et al. 1997; Kubbutat et al. 1997) . In the event of stress, p53 protein is phosphorylated, which leads to a much reduced affinity between p53 and MDM2, and thereby reactivating p53 (Jimenez et al. 1999). Nonetheless, MDM2 is constantly over-expressed in tumor cells, which significantly blocks the activation of p53 pathway even during stress conditions, thereby leading to the uncontrolled tumor cell proliferation (Momand et al. 1998). The overproduction of MDM2 makes tumors less susceptible to programmed cell death and apoptosis as a result of chemotherapy and other cancer therapy. Hence, the disruption of MDM2/p53 interaction in tumor cells should stabilize p53, preventing it from degradations, and initiating a cascade of p53 pathways to eventually sensitize the tumor cells to death (Murray et al. 2007). To date, targeting the MDM2/p53 interaction has become an emerging therapeutic approach in anticancer treatment. Numerous efforts have been taken for the development of inhibitors such as natural products (De Vincenzo et al. 1995; Stoll et al. 2001; Duncan et al. 2003; Tsukamoto et al. 2006), small molecules (Zhao et al. 2002; Galatin et al. 2004; Vassilev et al. 2004; Ding et al. 2005; Grasberger et al. 2005; Hardcastle et al. 2006), and oligomers (Alluri et al. 2003; Hara et al. 2006; Robinson 2008; Hayashi et al. 2009; Michel et al. 2009; Bautista et al. 2010). Compared to small molecule approach, oligomers are easily programmable and are readily synthesized by solid phase synthesis. It is also believed that the larger size of oligomers relative to small molecules may bring them additional advantages to contact more protein surface area, which will lead to enhanced binding affinity (Murray et al. 2007).

However, oligomers made of natural peptides are subjected to biodegradations and are also immunogenic in vivo, which limit their practical applications, but on the other hand underscore the need for unnatural peptides (Patch et al. 2002). Peptidomimetics are a class of non-natural peptide mimics using the artificial backbones to mimic peptides' primary and secondary structures (Wu et al. 2008). Compared to traditional peptides, peptidomimetics have great proteolytic and metabolic stability and are believed to be less immunogenic, also with an enhanced bioavailability (Patch et al. 2002; Goodman et al. 2007; Wu et al. 2008). The development of peptidomimetics to disrupt MDM2/p53 has led to a diverse set of oligomers such as β-peptides (Seebach et al. 1996; Cheng et al. 2001; Kritzer et al. 2005), γ- and δ-peptides (Arndt et al. 2004; Trabocchi et al. 2005; Kumbhani et al. 2006), α/β-peptides (Horne et al. 2008; Horne et al. 2009), azapeptides (Graybill et al. 1992; Lee et al. 2002), α-aminoxy-peptides (Li et al. 2008), sugar-based peptides (Risseeuw et al. 2007; Tuwalska et al. 2008), peptoids (Simon et al. 1992), oligoureas (Boeijen et al. 2001; Violette et al. 2005), polyamides (Dervan 1986), and phenylene ethynylenes (Nelson et al. 1997), etc. Nonetheless, the development of peptidomimetics is far less straightforward, with the major limit lying in the availability of framework (Goodman et al. 2007). The search for peptidomimeitcs of a variety of backbones remains crucial in the research of peptide mimics, which would result in different classes of oligomers with diverse structures and functions (Goodman et al. 2007;

Horne et al. 2008; Gellman 2009). The development of new peptidomimetics would also facilitate the identification of novel therapeutic agents and help the understanding of protein folding and functions by using peptidomimetic probes, all of which are important to the progress in modern chemical biology research (Goodman et al. 2007; Horne et al. 2008; Gellman 2009).

2. Development of AApeptides

In the attempt to search for new peptide mimics for drug discovery and protein mimicry, we recently described a novel class of peptidomimetics termed "AApeptides", which is derived from N-acylated-N-aminoethyl amino acids that has been previously used as the building block for PNA (Winssinger et al. 2004; Dragulescu-Andrasi et al. 2006; Debaene et al. 2007). Compared to natural peptides, the repeating unit of the AApeptide is structurally similar to two adjacent residues of α-peptide, in which there are two side chains, one from the regular α-amino acid side chain, while the other one from a carboxylic acid residue appended to the tertiary amide nitrogen. Depending on the relative position of α-amino acid side chain, there are two types of AApeptides. The one with α-amino acid side chain at the α position is called α-AApeptide (Hu et al. 2011), while the other one with side chain at the γ position is called γ-AApeptide (Niu et al. 2011) (Figure 1).

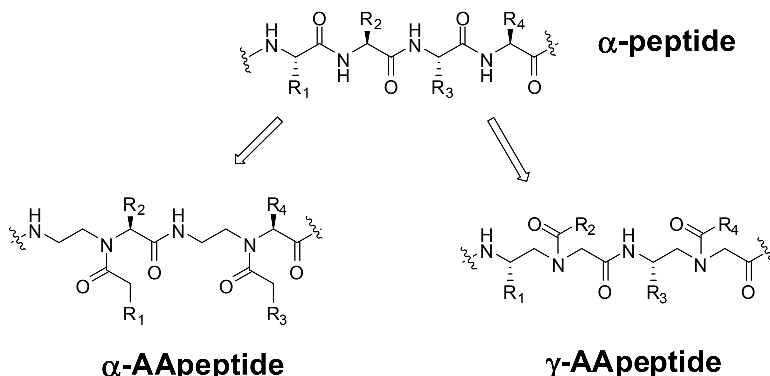


Fig. 1. Structures of a α-peptide and the corresponding AApeptides.

Both type of AApeptides project the same number of functional groups as conventional peptides with backbones of the same length. In addition, all the nitrogen atoms of AApeptides are involved in either secondary or tertiary amide bonds, in a way similar to natural α-peptide. Taken together, such AApeptides are designed to mimic the distance relationships and relative positions of amino acid side chains of natural peptides, so that they can reserve some functions of conventional peptides. It is also noteworthy that, even though AApeptides can mimic the structure as well as some activities of natural peptides, they are still different in backbone and should possess distinct hydrogen bonding properties and conformational flexibilities. The backbone of AApeptide is more flexible, with involved tertiary amide bonds potentially in cis/trans conformations, suggesting that the direct inter-conversion of sequences between AApeptides and natural peptides may not result in the same activity and functions.

3. Development of AApeptides for inhibition of p53/MDM2 interaction

For proof of concept, we demonstrated the facile synthesis and potential bioactivities of AApeptides by developing AApeptide based inhibitors of the p53/MDM2 model system, which has been a testing ground for freshly developed peptidomimetics of novel frameworks.

3.1 Design of AApeptide sequences

Previous reports indicated that synthetic agents displaying hydrophobic side chains of Phe19, Trp23, and Leu26, and in the orientation mimicking the array of these amino acids in p53 should compete with p53 in occupying the MDM2 cleft (Murray et al. 2007). Based on these findings, we designed four α-AApeptides and three γ-AApeptides to mimic the binding surface of p53 (Figure 2 and 3).

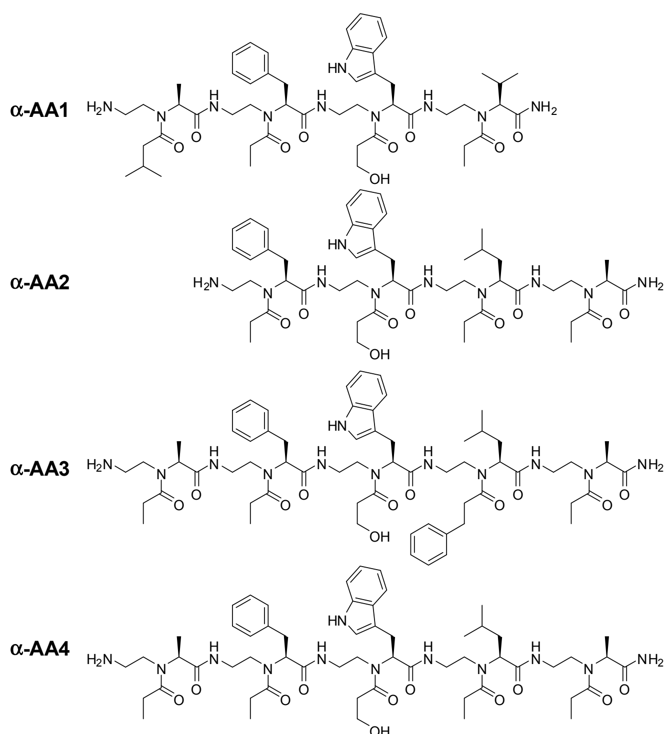


Fig. 2. α-AApeptide sequences designed for inhibition of p53 / MDM2 interaction. Figure is adapted from (Hu et al. 2011).

These AApeptides bear either some or all of the functional side chains of the three amino acids (Phe19, Trp23, and Leu26), which are designed to be the amino acid side chains at either α (for α-AApeptides) or γ positions (for γ-AApeptides). The other functional groups were randomly chosen, with most of them appended to the nitrogens of AApeptides through the formation of tertiary amide bonds.

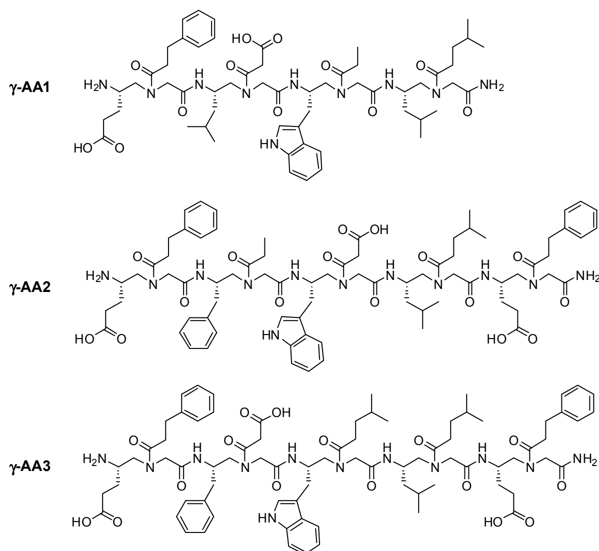


Fig. 3. γ-AApeptide sequences designed for p53/MDM2 disruption. Figure is adapted from (Niu et al. 2011).

3.2 Synthesis of AApeptides

In our initial attempt, we tried to synthesize AApeptides on solid phase resins through a direct sub-monomer strategy, in which the functional groups were introduced to the sequence step by step (Figure 4). Unfortunately, the presence of multiple secondary amines in the peptide backbone led to a constant over-alkylation during the reductive amination step. As a result, we only obtained a mixture of unidentified products after several coupling

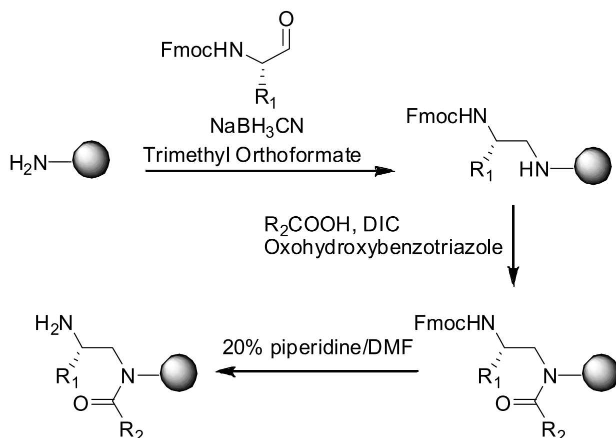


Fig. 4. Initial unsuccessful attempt to synthesize AApeptides on solid phase. Figure is adapted from (Niu et al. 2011).

cycles, as observed on HPLC after the cleavage of products from solid phase. We then carried out an alternative "monomer building block" strategy, in which building block was first synthesized in solution phase, and then assembled following the same procedure of standard solid phase synthesis of conventional peptides. In this route, AApeptide building blocks are readily prepared using commercially available agents at low cost.

For the synthesis of α-AApeptide building block (Figure 5), the carboxylic acid of amino acid was first protected to form the amino acid benzyl ester (**A**) or the amino acid tert-butyl ester (**B**). The resulting amino acid esters were then reacted with Fmoc-amino ethyl aldehyde by reductive amination to form secondary amines **2**, which were subsequently acylated with functional groups R^1. The coupling products **3** were finally deprotected with hydrogenlysis to remove the benzyl protecting group, or with trifluoroacetic acid to remove the tert-butyl protecting group. For the synthesis of γ-AApeptide building block (Figure 6), glycine benzyl ester was reacted with Fmoc-amino aldehydes through reductive amination, and the resulting intermediates **2** was subsequently acylated with carboxylic acid ended functional groups to form the coupled intermediate **3**. After a hydrogenation step, the desired γ-AApeptide building blocks **4** were finally obtained. For both α and γ AApeptides, a diverse set of conjugation conditions for the preparation of intermediates **3** were investigated. It was found that the use of activation agents such as HBTU/HOBt, DIC/HOBt, or PyBOP can provide the desired products in poor yields, and only when intermediate **2** was conjugated with a few types of carboxylic acids. After many trials, the coupling with oxohydroxybezotriazole / DIC emerged as the most efficient and can catalyze the successful conjugations of most carboxylic acids. It is also noteworthy that the derivatization of AApeptides is virtually limitless, since there are countless carboxylic acids available for acylation of nitrogen atom in the backbone. This specific feature allows the rapid generation of AApeptide library, which literally should have much more diversity than those libraries based on regular peptides, thereby expanding the versatility of oligomer libraries for potential applications in high-throughput screening based drug discovery and chemical biology research. With the prepared building blocks in hand, the solid phase synthesis of AApeptides was carried out on resins in a simple and highly efficient way. The sequences were finally obtained over 80% yield in crude and were purified by HPLC to achieve purities over 95%. Their identities were further confirmed by MALDI-mass spectrometry.

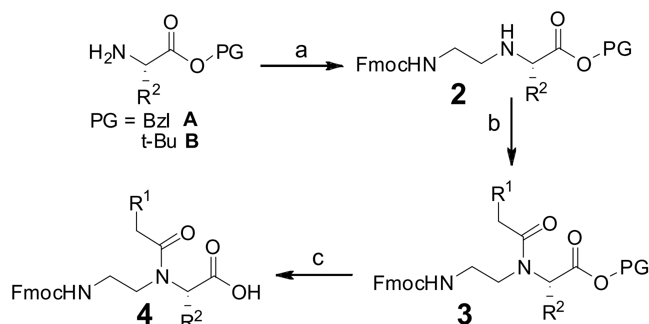


Fig. 5. Synthesis scheme of α-AApeptide building block. a) Fmoc-amino ethyl aldehyde, $NaBH_3CN$, overnight. b) R_1CH_2COOH, DhBtOH/DIC, overnight. c) Pd/C, H_2 for A; 50% TFA/CH_2Cl_2 for B. Figure is adapted from (Hu et al. 2011).

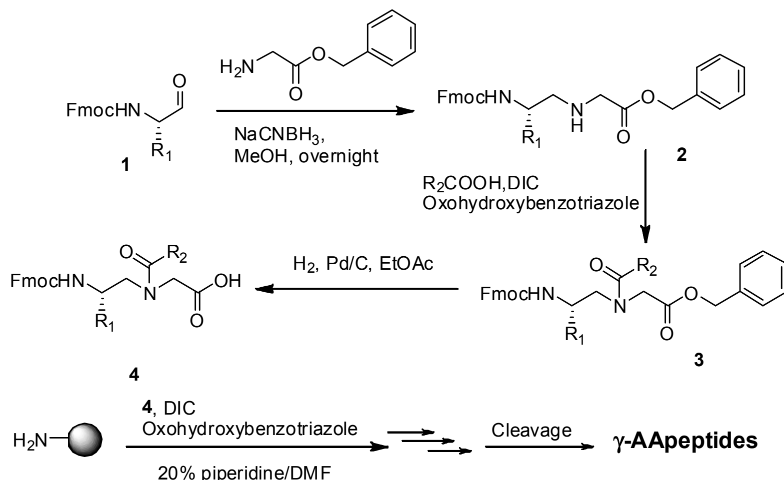


Fig. 6. Synthesis scheme of γ-AApeptide building block. Figure is adapted from (Niu et al. 2011).

3.3 ELISA assay of AApeptides for inhibition of p53/MDM2 interaction

These AApeptides were then tested by the ELISA assay for their inhibition of p53/MDM2 protein-protein interaction. Generally the ELISA plate was coated with p53, and then incubated with the mixture of MDM2 protein and AApeptide for one hour. The p53 bound MDM2 protein was then detected by MDM2 antibody and a secondary antibody conjugated with the horseradish peroxidase, which later on reacted with the TMB peroxidase substrate to manifest a yellowish color after acid quenching. The color intensity was monitored by absorbance at 450nm, the extent of which is directly proportional to the amount of bound MDM2 protein, and also reversely correlated to the inhibiting efficiency of AApeptide. The readings for each sample were then plotted against the concentration of α-AApeptide (Figure 7) or γ-AApeptide (Figure 8). Almost all AApeptides inhibit the p53-MDM2 binding when they are administrated at high concentrations.

Based on the plots of each peptide in Figure 7 and 8, the related IC_{50} values were calculated and summarized in Table 1. The published IC_{50} value of the wide type p53-drived peptide (Garcia-Echeverria et al. 2000) was also included for comparison. For α-AApeptide, **α-AA4** is the most prominent one, with an IC_{50} of 38μM, which is comparable to the previously reported β-peptides and peptoids (Knight et al. 2002; Kritzer et al. 2004; Hara et al. 2006), and is only 4-5 fold less potent than the reported p53-drived wild type peptide (Garcia-Echeverria et al. 2000). Consistent to previous reports (Kussie et al. 1996), the preliminary structure and activity relationship (SAR) study here suggests that the inclusion of functionalities of the three key amino acids "Phe, Typ, and Leu" are important to maintain the strong binding affinity, which are present in all sequences but **α-AA1**. Compared to **α-AA2**, the change of Leu to Val in **α-AA1** decreases the binding affinity to at least 10-fold. Further, that observation of **α-AA4**'s much higher activity than **α-AA2** indicates that better activities are possessed by longer sequences. The longer sequence may possibly have a better stabilized backbone conformation.

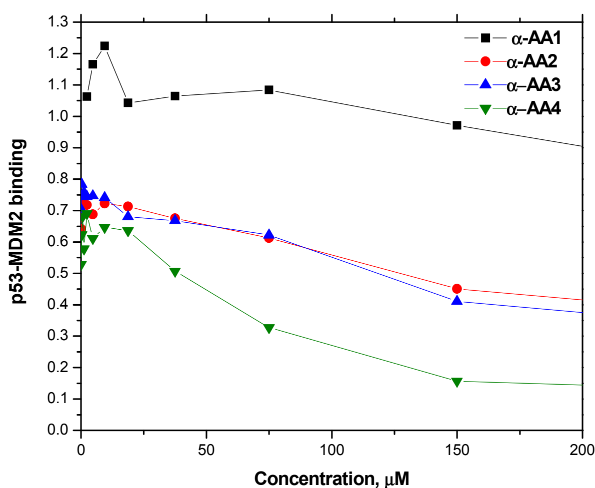


Fig. 7. Plots of ELISA assay for the inhibition of p53-MDM2 interaction by α-AApeptides Figure is adapted from (Hu et al. 2011).

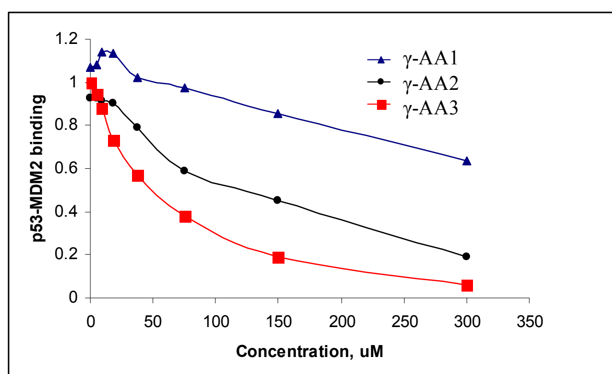


Fig. 8. Plots of ELISA assay for the inhibition of p53-MDM2 interaction by γ-AApeptides. Figure is adapted from (Niu et al. 2011).

AApeptides	IC_{50} (μM)
α-AA1	>1000
α-AA2	120 ± 10
α-AA3	120 ± 16
α-AA4	38 ± 8
γ-AA1	> 400
γ-AA2	120 ± 15
γ-AA3	50 ± 8
p53-derived peptide (Ac-QETFSDLWKLLP)	8.7

Table 1. ELISA results of AApeptides for the disruption of p53/MDM2.

Finally, since **α-AA4** differs from **α-AA3** in only one residue, the side chains not involved in the recognition of MDM2 may also play a substantial role in the binding event. In this case, the Phe side chain of **α-AA3** may be either in the hydrophobic binding cleft, or near the binding domain, clashing with the residues of MDM2 and thereby raising up their binding energy. For γ-AApeptides, **γ-AA3** turns out to be the most effective inhibitor, with an IC_{50} value of 50μM, which is comparable to the most active α-AApeptide and is also only a few fold less active than p53-derived peptide (Garcia-Echeverria et al. 2000). Compared to the others, replacement of Phe by Leu in **γ-AA1** peptide results in a significant loss of activity. Whereas **γ-AA2** and **γ-AA3** both have the required "Phe, Typ, and Leu", the slight difference of their side chain functionalities between Phe and Leu results in more than two fold difference in activity, suggesting that even the side chains are also involved in the binding pocket. This observation is similar to the situation of α-AA peptides.

3.4 Computer modelling for bioactive AApeptides

The ELISA results were further confirmed by preliminary computer modeling studies (Figure 9 and 10), which shows that the side chains of Phe, Trp and Leu in the energy-minimized structures of both **α-AA4** and **γ-AA3** are able to overlap perfectly with those residues in the helical domain of natural peptide p53, indicating that **α-AA4** and **γ-AA3** should be able to mimic the recognition of p53 to MDM2 very well. Compared to **γ-AA3**, **α-AA4** appears to prefer an extended conformation when interacting with MDM2.

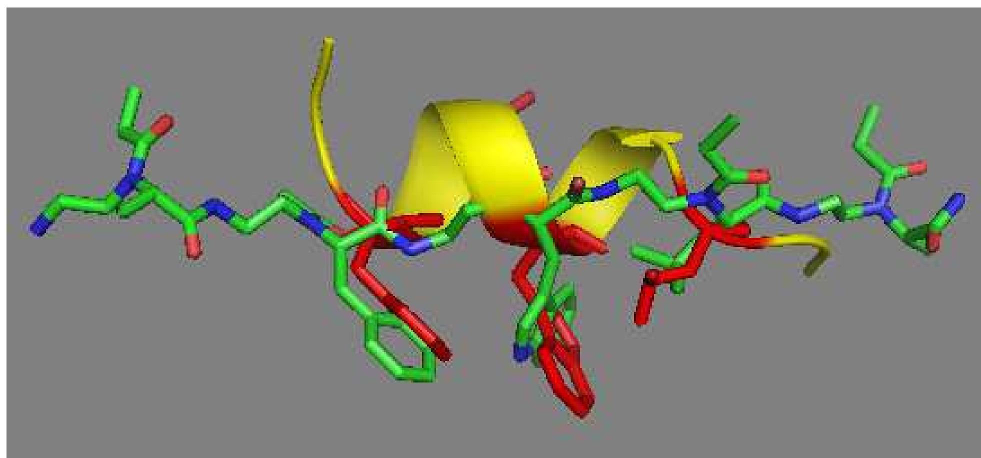


Fig. 9. Energy minimized (MM2) structures of **α-AA4** (green colored) and amino acids 17-29 of p53 helical domain (yellow colored). **α-AA4** is shown as sticks, and the three critical residues (Phe19, Trp23, and Leu 26) in p53 responsible for binding to MDM2 are also presented in sticks and colored in red. Figure is adapted from (Hu et al. 2011).

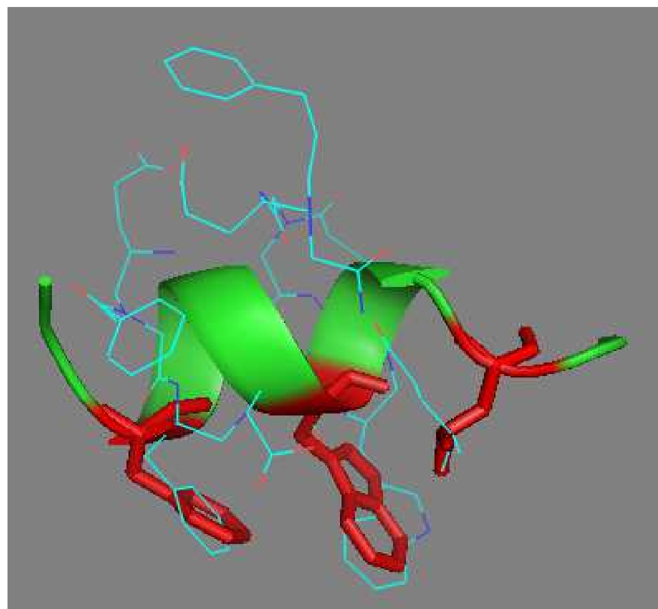


Fig. 10. Energy minimized (MM2) structures of **γ-AA3** (blue colored) is superimposed with the amino acids 17-29 of p53 helical domain (green colored). Three critical residues (Phe19, Trp23, and Leu 26) in p53 responsible for binding to MDM2 are presented in as sticks and colored in red. **γ-AA3** is shown as the wire frame presentation. Figure is adapted from (Niu et al. 2011).

3.5 Summary

Taken together, these results demonstrated AApeptides as a novel class of peptidomimetics. It is also noteworthy that both classes of AApeptides bear excellent selectivity, with different sequences giving different activities, instead of a random interaction with proteins. For example, **α-AA4** is the strongest inhibitor among all the demonstrated α-AApeptides, while **α-AA1** is a poor inhibitor, and **α-AA2**, **α-AA3** are weak inhibitors. Similarly, **γ-AA3** appears to be the best inhibitor, while **γ-AA1** turns out to be the worst.

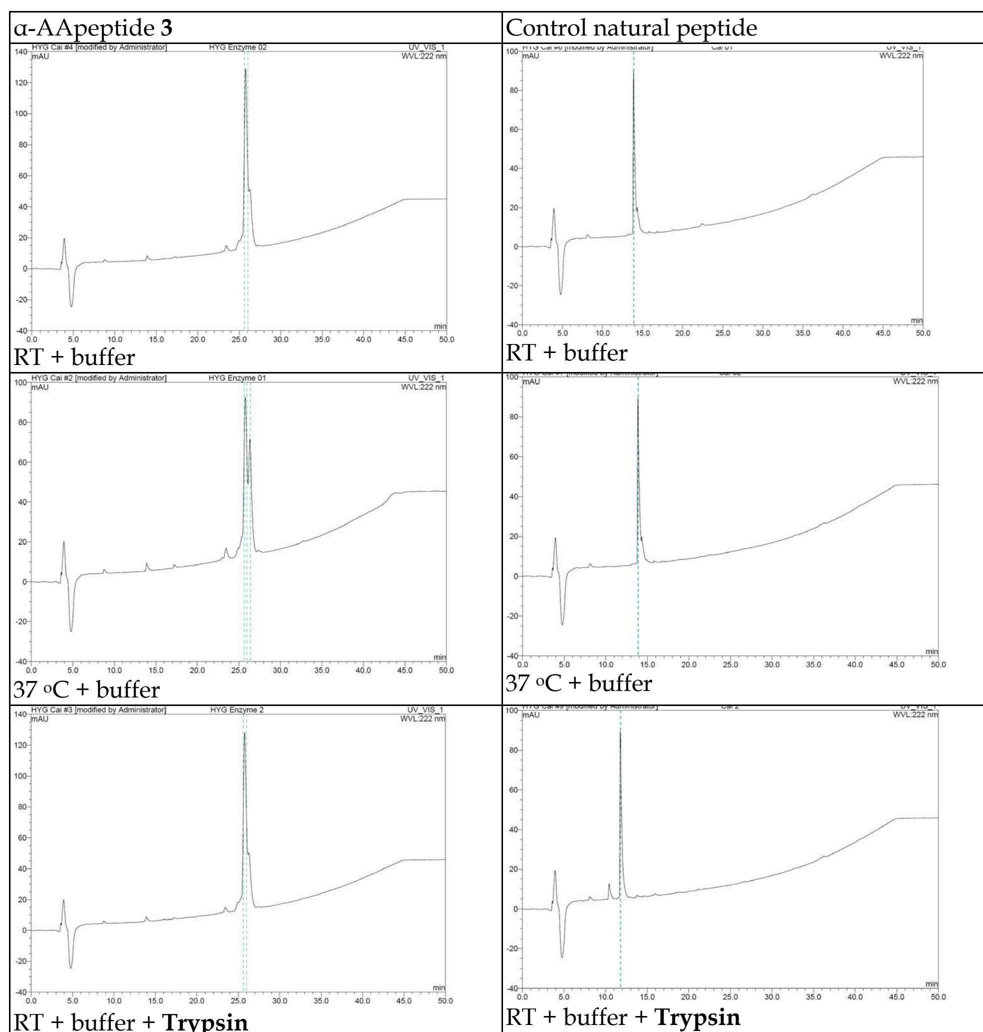
Detailed structure-activity relationship studies for AApeptides with various lengths and distribution of functional groups along the backbone are currently ongoing, which should provide valuable information for rational design of AApeptide library for drug discovery and chemical biology research. Generally, much more potent AApeptide derivatives are expected with the stabilization of secondary structure, introduction of halogen atoms, and computer modeling-aided design (Hara et al. 2006; Murray et al. 2007; Michel et al. 2009).

4. Stability of AApeptides

One significant advantage of peptidomimetics is the superior resistance to proteolysis, owing to their unnatural backbones. To find out the stability of our AApeptides in this regard, representative sequences of α- and γ- AApeptides (**α-AA3**, **γ-AA3**) were incubated with proteases at the concentration of 0.1 mg/mL in 100 mM pH 7.8 ammonium bicarbonate

buffer for 24 hours. **α-AA3** was mixed with chymotrypsin, trypsin, and pronase, respectively; and **γ-AA3** was mixed with chymotrypsin, thermolysin, and pronase, respectively. All the reaction mixtures were then analyzed by HPLC. The retention time and integrations of eluted peaks were compared with those of peaks representative of the starting materials.

As shown in figure 11 and figure 12, whereas conventional peptides are susceptible to proteolysis, especially by chymotrypsin and pronase, both **α-AA3** and **γ-AA3** are highly resistant to enzymatic hydrolysis within 24 hours. There are, however, a small shoulder observed for both types of AApeptides at 37°C with or even without incubation with proteases, which takes up less than 5% of the total volume and is presumably due to the isomerization of the syn/anti tertiary amide bonds in the peptide backbones.



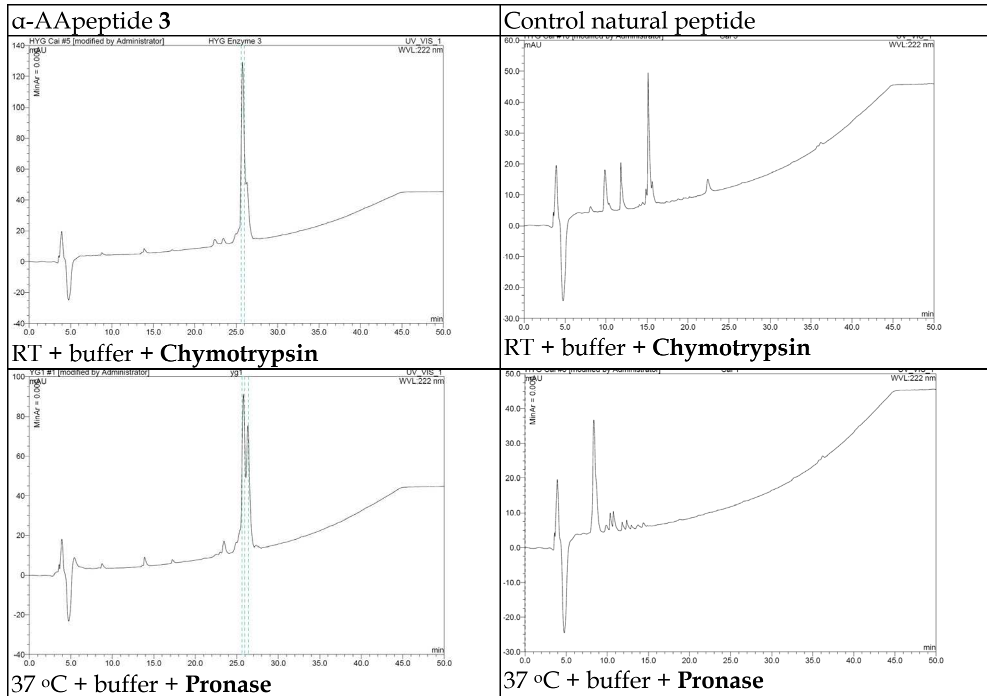
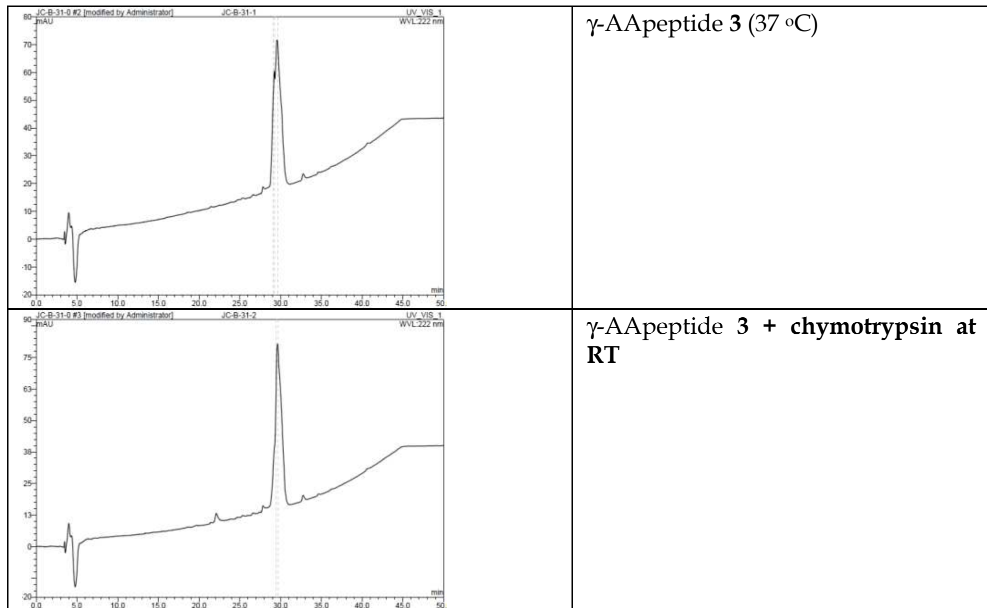


Fig. 11. Analytical HPLC spectra of **α-AA3** and control α-peptide after their incubations with different proteases. Figure is adapted from (Hu et al. 2011).



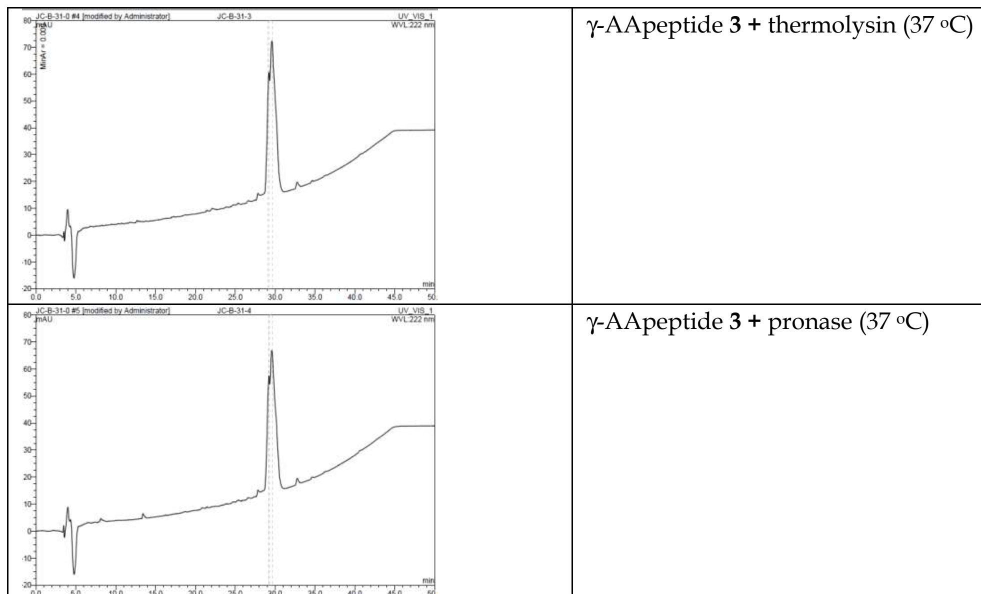


Fig. 12. Incubation of **γ-AA3** with different proteases. Figure is adapted from (Niu et al. 2011).

5. Conclusions

In conclusion, we have developed a new class of peptidomimetics – the AApeptides that can have amino acid side chains at either α-, or γ-position. This family of peptides can be readily synthesized on solid phase by standard monomer building block approach, using α or γ-substituted N-acylated-N-Fmoc-amino ethyl amino acid building blocks. Given the availability of countless types of carboxylic acids, the AApeptides are amenable to potential derivatizations with a wide variety of side chains in a simple and straightforward manner, indicating its promising applications in library based drug screening. The preliminary results show that AApeptides possess significant bioactivities including the mimicry of p53 to successfully inhibit the p53/MDM2 protein-protein interaction, the selectivity in binding MDM2 protein, and the excellent stability towards enzymatic degradations. Hence, it is conceivable that a continuing development of sequence-specific AApeptides would enrich the current types of functional peptidomimetics, and expand the applications of peptide mimics in biomedical research including the modulation of protein-protein interactions. Future work will involve the systematic studies using X-ray crystallography, Circular Dichroism (CD), and 2D-NMR to understand the structure requirements of AApeptides to adopt predicted conformations, which will help the design of functional AApeptides. More specifically, the optimizations of AApeptide sequences to achieve a better inhibition of p53/MDM2 interaction as well as other carbohydrates/proteins/nucleic acids interactions are also urgent and are currently under investigation.

6. Acknowledgement

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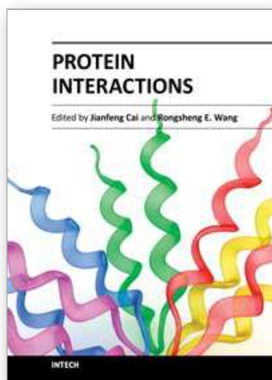

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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