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**Smac-Derived Aza-Peptide As an Aminopeptidase-Resistant XIAP BIR3 Antagonist**

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**Abstract:** The peptidic nature of anti-IAPs N-terminus Smac-derived peptides precludes their utilization as potential therapeutic anticancer agents. Recent advances in the development of novel Smac-derived peptidomimetics and non-peptidic molecules with improved anti-IAPs activity and resistance to proteolytic cleavage have been reported and led to a number of candidates that are currently in clinical trials including LCL-161, SM-406/AT-406, GDC-0512/GDC-0917, and birinapant. As an attempt to improve the proteolytic stability of Smac peptides, we developed the Aza-peptide AzaAla-Val-Pro-Phe-Tyr-NH2 (1). Unlike unmodified peptide Ala-Val-Pro-Phe-Tyr-NH2 (2), analogue (1) exhibited resistance towards proteolytic cleavage by two aminopeptidases, LAP and DPP-IV, while retaining its IAP inhibitory activity. This was due to the altered planar geometry of the P1 residue side chain. Our findings showed that using aza-isosteres of bioactive peptide sequences imbue the residue with imperviousness to proteolysis; underscoring a potential approach for developing a new generation of Smac-derived Aza-peptidomimetics.

**Keywords:** Aminopeptidases, Apoptosis, Aza-peptides, Caspase 9, IAPs, Smac.

**INTRODUCTION**

Inhibition of the anti-apoptotic IAPs proteins by short peptides derived from the N-terminal sequence of the pro-apoptotic mitochondrial protein Smac is an emerging strategy for re-engaging apoptosis in resistant cancer cells overexpressing IAPs. Although Smac-derived peptides have been demonstrated to augment the activity of anticancer drugs both in-vitro and in-vivo [1, 2], their entirely peptidic nature almost certainly precludes their utilization as anticancer therapeutic agents. This is because of the innate and generally impaired bioavailability of peptide ligands mainly due to their instability towards proteolytic cleavage. There have been long standing efforts in developing protease-resistant Smac-derived peptidomimetics (monovalent and bivalent) and non-peptidic analogues by ourselves [3] and many other groups [4-9] that led in some cases to IAP inhibitors that reached clinical trials [10] such as LCL-161, SM-406/AT-406, GDC-0512/GDC-0917, and birinapant. As an attempt to improve the proteolytic stability of Smac peptides, we developed the Aza-peptide AzaAla-Val-Pro-Phe-Tyr-NH2 (1). Unlike unmodified peptide Ala-Val-Pro-Phe-Tyr-NH2 (2), analogue (1) exhibited resistance towards proteolytic cleavage by two aminopeptidases, LAP and DPP-IV, while retaining its IAP inhibitory activity. This was due to the altered planar geometry of the P1 residue side chain. Our findings showed that using aza-isosteres of bioactive peptide sequences imbue the residue with imperviousness to proteolysis; underscoring a potential approach for developing a new generation of Smac-derived Aza-peptidomimetics.

Since the acylation of the amino function of the P1 Ala residue of Smac abolished the binding affinity of the Smac peptide to XIAP BIR3 [19], as did N,N-dimethylation [4], such structural modifications cannot be applied for hindering the possible exposure of the N-terminal segment of the peptide to proteolytic cleavage. It is worth mentioning here that only the N-termiinally mono-methylated (N-methyl) Smac-derived peptides have preserved IAP inhibitory activity [4], and similar modification is thus currently widely used as an aminopeptidase resistant residue at P1 in the Smac-derived peptidomimetic and in non-peptidic clinical candidates [11-18].

Based on the above discussed SAR, we have investigated the replacement of the N-terminal alanine of peptide (1), with aza-alanine (AzaAla) residue. Many studies demonstrated that the incorporation of aza-amino acids into susceptible peptide cleavage sites in peptides renders them less likely to be hydrolysed and thus enhancing the peptide sequence metabolic stability, biological action and elongating the duration of action [21]. We had anticipated that the...
modified peptide AzaAla-Val-Pro-Tyr-NH₂ (2) could still make the important electrostatic contacts within the XIAP BIR3 binding pocket, by virtue of the retained and unmodified primary amino function of the AzaAla residue, whilst exhibiting enhanced stability towards aminopeptidases (exemplified mainly by LAP). All this is due to the altered configuration at P1, where the side chain residue of the SP² hybridised L-amino acid (Fig. 2) was formed, which was filtered, washed with ethyl acetate and dried under vacuum to afford white powder of melting point 145-150 °C. 1H NMR (CDCl₃, 400 MHz) 6 7.94-7.32 (m, 8H), 6.24 (br s, 1H), 4.40 (d, 2H), 4.16 (t, 1H), 2.58 (s, 3H); 13C NMR (CDCl₃, 100 MHz) 6 157.13, 143.73, 141.36, 127.76, 127.07, 124.97, 120.01, 67.01, 47.24, 39.29; ESI-Mass for sample dissolved in methanol m/z 291.3 [M + Na]+.

Coupling of 1-chlorocarbonyl-2-(9H-Fluoren-9-methoxy carbonylamino)-1-methylhydrazine (6) to Val-Pro-Phe-Tyr-Resin (7) (Scheme 1, Route A)

To a 50 mL round bottom flask, containing a cold solution (-10°C) of triphenylphosphine (0.66 mmol, 196 mg) and 2-(9H-Fluoren-9-methoxy carbonylamino)-1-methylhydrazine (5) (2 mmol, 536 mg) in 15 mL THF, was added drop wise N-methylmorpholinium chloride was formed. The reaction mixture was stirred at -10°C for 8 hours and then concentrated under vacuum, 1-chlorocarbonyl-2-(9H-Fluoren-9-methoxy carbonylamino)-1-methylhydrazine (6) residue 500 mg (1.5 mmol, 75% yield) was obtained, ESI-Mass for sample dissolved in methanol m/z 328.5 [Fmoc-NH-Ni(CH₃)₂COOCH₃] THF. The residue was then used for the microwave-assisted coupling to H₂N-Val-Pro-Phe-Tyr-Resin (7) with DEA as activator base, where 1-chlorocarbonyl-2-Fmoc-1-methylhydrazine (DEA 1/2). The reaction was repeated for 7 cycles (coupling completion was monitored by Kaiser Test); each coupling cycle was performed for 10 minutes, at 22 Watts, at a temperature of 75 °C. After coupling completion, the peptide was cleaved from the resin and purified by RP-HPLC as previously described [3].
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Caspase-9 Activity Fluorimetric Assay

MDA-MB-231 cell lysate was prepared by solubilising cells pellet (~ 5 x 10⁷ cells) in 1 mL ice cold lysis buffer (50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 0.2% (w/v) CHAPS and 50 mM HEPES; pH 7.4), supplemented with a protease inhibitor cocktail (50 µg/mL Antipain,2HICh, 40 µg/mL Bestatin, 6 µg/mL Chymostatin, 7 µg/mL Pepstatin, 50 µg/mL Phosphoramidon, 1 mg/mL Pefabloc SC, 0.5 mg/mL EDTA-Na₂ and 2 µg/mL Aprotinin), and was incubated on ice for 15 min. Cytochrome c (5 µg; 1 mg/mL) and dATP (5 mM) in assay buffer (10 mM HEPES, 0.5 mM EGTA, 5 mM DTT and 10% (v/v) glycerol; pH 7.4) were added to 25 µL cell lysate into a 1.5 mL microcentrifuge tube (25 µL of assay buffer was used instead for blank) with/without 1 µM XIAP HIR3 (500 mM final concentration) and with/without 0.5 µL treatment peptide in DMSO (final concentrations of 1 µM), which were then incubated at 37°C for 1 h. Each reaction mixture was then diluted with 65 µL assay buffer; all were transferred to 96-well microtiter plate and then 5 µL of Ac-LEHD-AFC substrate (50 µM final concentration) were added per well. A FLUOSTAR OPTIMA spectrofluorimeter (BMG LABTECH, Ortenberg, Germany) was used at excitation wavelength of 380-400 nm and emission wavelength of 470-500 nm to determine the caspase-9 activity through the Ac-LEHD-AFC substrate cleavage with the release of 7-amino-4-trifluoromethylcoumarin fluorescent moiety. The reactions were monitored for 1 h. Reactions were carried out in triplicates for accuracy. Finally, percent caspase-9 release was calculated from the following formula:

\[
\% \text{ caspase-9 derepression} = 100 \times \left( \frac{\text{slope of control curve}}{\text{mean value of control curve} + \text{mean value of test curve}} \right)
\]

RESULTS AND DISCUSSION

Synthesis of AzaAla-Val-Pro-Phe-Tyr-NH₂ (2)

The Microwave-assisted solid phase synthesis (MW-SPPS) of the target peptide included the coupling of 2-(9H-Fluoren-9-methoxycarbonylamo)-1-methylhydrazine (5) to the tetrapeptide sequence Val-Pro-Phe-Tyr-tethered to the resin (7) (Scheme 1), but first the synthesis of (5) was required. Compound (5) was synthesized from 1-(tert-butoxycarbonyl)-1-methylhydrazine (3), using the previously reported synthesis protocol by Busnel et al. [22] and others [23-25]. The synthesis comprises two steps, an initial Fmoc-protection of the primary amino function of the hydrazine, followed by Boc removal of the secondary amine functionality (Scheme 1). The introduction of the Fmoc-group was accomplished using Fmoc-OSu, in the presence of sodium bicarbonate, to obtain (4), which was then treated with TFA in order to effect the removal of the Boc-group. The final product, N-Fmoc-methylhydrazine (8), was obtained in high yield (85%, based on the starting amount of 4). The synthesis of the target Aza-peptide (2) was achieved as shown in Route A (Scheme 1) through the activation of (5) by treatment with triphosgene in the presence of N₁-methyl morpholine, at -10°C, in THF. The product, 1-chloro-carbonyl-2-(9H-Fluoren-9-methoxycarbonylamino)-1-methylhydrazine (6) was obtained in high yield (75%, based on the amount of starting material 5) and was used directly to acylate the resin-bound His₅-Val-Pro-Phe-Tyr-sequence (7) using microwave-assisted coupling, employing DIAE as activator base (Scheme 1, Route A). Samples of resin were subjected to the Kaiser Test and ESI-MS, in order to assess the extent of coupling (6). It was found that seven repeat coupling cycles (10 minutes/cycle, 22 Watts, 75°C) were required for completion of the reaction.

Boeglin and Lubell have previously shown that coupling of various Fmoc-aza-amino acid chlorides (including the N-terminus amino group of resin tethered peptides is only complete after 6 h reaction in DCM with DIAE as activator base using conventional SPPS, and in some cases the coupling has to be repeated to ensure completion [24]. Employing microwave-assisted coupling, we managed to significantly reduce the coupling reaction time to 70 minutes (7 cycles x 10 minutes/cycle). Unfortunately, even with this repetitive coupling protocol, (2) could only be obtained as a low purity (~25%) crude product, as estimated by analytical...
Scheme 1. On-resin microwave-assisted coupling of 2-(9H-fluoren-9-methoxycarbonylamino)-1-methylhydrazine (5) for the synthesis of AzaAla-Val-Pro-Phe-Tyr-NH₂ (2); triphosgene activation (Route A), p-nitrophenyl chloroformate activation (Route B) and carbonyldiimidazole activation (Route C).

RP-HPLC (data not shown). This could be due to the infeasibility of purifying the highly active and unstable intermediate (6), which necessitates using it as a crude product for coupling to the resin bound peptide (7). However, the crude Aza-peptide (2) was purified successfully, by semi-preparative RP-HPLC, to give a final product with a yield of only 8%, but of 98% purity (Fig. 3).

Triphosgene is not compatible for use within a microwave-assisted synthesizer, due to it being highly reactive and with potential for interaction with the amide groups of the microwave compatible solvents DMF and NMP. Therefore in attempts to improve the yield and purity of the product and to automate the synthesis, additional carboxylating agents were tried for coupling N-Fmoc-methylhydrazine (5)
to the N-terminal amino group of the resin-tethered peptide (7), under microwave conditions. We tried both para-nitrophenyl chloroformate (Scheme 1, Route B) and carbonyldiimidazole (Scheme 1, Route C) mediated carbonylation and coupling using the conditions described in the experimental section. Disappointingly, we did not obtain any of the target peptide, even after 7-repeat coupling cycles in case of para-nitrophenyl chloroformate (data not shown) and incomplete coupling was observed when carbonyldiimidazole was used as activator, as indicated by ESI-MS analysis (Fig. 4). Similar observations have been reported by Han et al., in the attempted incorporation of two aza-amino acids into target sequences when using para-nitrophenyl chloroformate and carbonyldiimidazole [26]. This could be due to a low inherent reactivity of the respective aryl carbamates and N-carbonyl imidazoles, or the poor accessibility of these derivatives to the amino functionality of the tethered peptide within the resin matrix, as reported by André et al. [27]. Ultimately, the AzaAla-Val-Pro-Phe-Tyr-NH₂ (2) synthesis was only successful through the triphosgene-mediated carbonylation approach (Scheme 1, route A).

Aminopeptidase Susceptibility

Compound (2) was then tested for its stability against two aminopeptidases; namely LAP and DPP-IV, and in comparison to the wild-type Smac-derived native sequence Ala-Val-Pro-Phe-Tyr-NH₂ (1) and the N-methyl alanyl analogue N-Me-Ala-Val-Pro-Phe-Tyr-NH₂ (9).
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Figure 4. ESI-MS trace for crude AzaAla-Val-Pro-Phe-Tyr-NH$_2$ (2) produced by carbonyldiimidazole-mediated carbonylation of N-Fmoc-methylhydrazine (5) showing peaks of unreacted Val-Pro-Phe-Tyr-NH$_2$ even after 7-repeated coupling cycles, indicating incomplete coupling.

Figure 5. RP-HPLC profiles of (a), Smac-derived pentapeptide analogue (1) incubated with LAP, for 30 min, at 37°C (b), AzaAla-analogue (2) and (c), N-Me-analogue (9) incubated with LAP, for 6 h, at 37°C.

LAP is an aminopeptidase that can cleave N-terminal Ala residues from peptides [28]. As anticipated, the P1 Ala residue was completely hydrolysed from the native peptide sequence (1) in 30 minutes, to give the truncated hydrolysis product Val-Pro-Phe-Tyr-NH$_2$, as shown by RP-HPLC and ESI-MS (Fig. 5 (a)). Strikingly, the AzaAla-analogue (2) was completely resistant to hydrolysis after 6 h of exposure to LAP (Fig. 5 (b)), as was the case with the N-methyl alanyl protected analogue (9) (Fig. 5 (c)). These results suggest that designing Smac-derived analogues with a planar SP$^2$ hybridized Aza-alanyl modified N-terminal will improve the proteolytic resistance for those candidates, making them as resistant as the widely used N-methyl alanyl congeners.

Interestingly, the Smac-derived pentapeptide analogue (1), AzaAla-analogue (2) and N-Me-Ala-analogue (9) proved to be resistant to aminopeptidase DPP-IV after 6 h exposure to the enzyme, as observed by RP-HPLC and ESI-MS (data not shown). Although DPP-IV is an N-terminal dipeptidyl
peptidase that can cleave a dipeptide unit in ‘one bite’ from longer peptides with Val at the P2 position [29], it might have been expected that all the three Smac-derived peptides (1), (2) and (9) would have been subject to degradation by this peptidase (they all have Val at P2). However, the presence of Pro at P3 makes all of the N-terminal Smac-derived sequences intrinsically resistant to DPP-IV, since peptides with Pro at P3 are known to function as DPP-IV inhibitors [30].

IAP Antagonistic Activity

Subsequently, the antagonistic activity of AzaAla-analogue (2) against XIAP BIR3 was assessed by caspase-9 activity recovery assay. In this assay, the ability of the Smac-derived analogues to reactivate caspase-9 in the presence of its inhibitor rhXIAP BIR3 (500 nM) was determined by the increase in caspase-9 activity that they induced.

Remarkably, the Aza-peptide analogue (2) retained the ability to antagonise the inhibitory effect of XIAP BIR3 on caspase-9 activation as effective as the wild-type (1) and the N-Methyl-Ala-sequence (9) (Fig. 6). In essence, (2) was able to restore 36.2 ± 4.9% of caspase-9 activity in the presence of 500 nM rhXIAP BIR3, at 1 μM compared to 43.1 ± 5.2% for the wild sequence (1) and 51.4 ± 7.1% for the N-Me-Ala-analogue (9), at the same concentration (mean ± SD, n=3) (Fig. 6). These results demonstrate that the planar conformation of the SP2 hybridised AzaAla residue did not dramatically miss-locate the side-chain methyl group at P1 permitting the essential hydrophobic interaction with the indole ring of the W310 residue of XIAP BIR3 (Fig. 1). Moreover, analogue (2) can still form the essential electrostatic interaction with the proximal Glu314 of XIAP BIR3, owing to the retained and unmodified N-terminal primary amino function of the AzaAla residue (Fig. 1).

CONCLUSION

In the light of the above results, the modified peptide AzaAla-Val-Pro-Phe-Tyr-NH₂ (2) was a successful bioisostere for the N-terminal Smac-derived pentapeptide parent sequence Ala-Val-Pro-Phe-Tyr-NH₂ (1). The Aza-peptide (2) is an equipotent analogue for (1) as a XIAP BIR3 inhibitor, whilst exhibiting enhanced stability towards aminopeptidases, exemplified mainly by LAP, owing to the planar geometry of the Aza-Ala residue at P1. Although the reported Aza-peptide (2) is not a therapeutic candidate per se, due to lack of cell permeability, it represents an important lead compound for the development of a new class of anti-cancer Smac-derived Aza-peptidomimetic therapeutic candidates. Our current studies focus on developing libraries of these novel candidates, where the N-terminal Ala residue at P1 is occupied with the Aza-Ala isostere instead of the widely used N-methyl substituted Ala, with P2, 3 and 4 residues modified as previously reported by ourselves [3] and others [4]. These structural modifications of the amino Smac-derived Aza-peptidomimetics are expected to improve the efficacy and proteolytic stability of these therapeutic candidates.

LIST OF ABBREVIATIONS

IAPs = Inhibitor of Apoptosis Proteins
Smac = Second mitochondria-derived activator of caspases
XIAP BIR3 = X-linked Inhibitor of Apoptosis Protein-Baculovirus IAP Repeat-3 domain
SAR = Structure Activity Relationship
LAP = Leucine Aminopeptidase
DPP-IV = Dipeptidyl Peptidase-IV

CONFLICT OF INTEREST

The authors declare there is no conflict of interest for this research work.

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