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Synthesis and biological evaluation of modified pentapeptides as potent proteinase K inhibitors

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ABSTRACT

This communication reports the first demonstration of synthesis and biological validation of modified pentapeptides, such as methoxysuccinyl-Ala-Ala-Ala-Pro-Leu-chloromethyl ketone **6b** as a potent proteinase K inhibitor. The efficacy of MeOSuc-AAAPL-CH_2Cl **6b** analog in inhibiting the proteolytic activity of proteinase K was compared with the known MeOSuc-AAPV-CH_2Cl analog. The examination of inhibitory activity using RT-PCR assay in the presence of proteinase K revealed that the MeOSuc-AAAPL-CH_2Cl **6b** inhibitor at a concentration of 0.05 mM allows a signal to be obtained for an exogenous target ('Xeno RNA') at 30 cycles (i.e., Ct = 30), whereas the control MeOSuc-AAPV-CH_2Cl requires a fivefold higher concentration (0.25 mM) to produce the same Ct. A plausible explanation for the higher efficiency of MeOSuc-AAAPL-CH_2Cl **6b** over control is proposed based on the molecular modeling studies.

The inhibition of proteases by specifically designed molecules is of particular interest in view of the involvement of these enzymes in biological processes which frequently are of medical importance.[1] The peptide chloromethyl ketones have been more intensively studied as a inhibitors for two reasons: (i) they bind covalently to the catalytically active histidine and serine residues in the ubiquitous serine proteases and (ii) their peptide sequence can be synthesized according to the substrate specificity of the target enzyme.[2–4] Currently, peptide-linked halomethyl ketones are probably the most widely-used class of affinity labels.[5] The target serine proteases are involved in a number of important physiological processes including blood coagulation, the complement system, fertilization, and protein turnover. Many of these enzymes are believed to be involved in diseases such as emphysema, arthritis, and tumorogenesis. Selective regulation of these and other proteases in the disease state is an important clinical problem.[6] In continuation of our search efforts[7] to find better inhibitors to eliminate the need for proteinase K removal prior to further in vitro enzymatic reactions, we set out to evaluate the application of newly synthesized modified pentapeptides. The modified pentapeptides are designed in such a way as to provide a methoxysuccinyl group at the N-terminus and a chloromethyl ketone group at the C-terminus of the penta-amino acid chain. From the X-ray crystallographic studies,[8,9] it appears that the presence of the methoxysuccinyl group aids binding to the recognition site of proteinase K by two hydrogen bonds, one directly between the methoxy oxygen of the succinyl group and the hydrogen of the Tyr^{104} (N), and the other mediated by a water molecule, succinyl-peptide (C+O)···Wat^{453}···(HN)Gly^{136}. In this Letter, we report the first example of the synthesis and proteolytic inhibitor function of pentapeptides such as MeOSuc-AAAPV-CH_2Cl **6a** and MeOSuc-AAAPL-CH_2Cl **6b**.

The synthesis of the desired pentapeptides MeOSuc-AAAPV-CH_2Cl **6a** and MeOSuc-AAAPL-CH_2Cl **6b** is depicted in Scheme 1. The peptide coupling reaction of Boc-AAAP-OH **1** with Val-CH_2Cl.HCl **2a** via the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine using THF as the solvent afforded the corresponding Boc-AAAPV-CH_2Cl **3a** in 95% yield. The cleavage of Boc group was accomplished using 6 M HCl/EtOAc system to afford the corresponding H-AAAPV-CH_2Cl.HCl **4a** in 96% yield. Finally, treatment of **4a** with methyl succinimido succinate **5** in the presence of THF/$NaHCO_3$ system furnished corresponding **6a** in 82% yield. A similar synthetic strategy was performed to obtain the corresponding pentapeptide **6b**. The resulting modified pentapeptide structures of **6a** and **6b** were characterized and confirmed by ^1H NMR and mass spectroscopy.[10,11]

Reverse transcription-polymerase chain reaction (RT-PCR) with real-time detection of amplification products is a robust, simple, and quantitative way to measure RNA levels in solution. These measurements can be made directly from cultured cells using the Cells-to-CT™ kit from Applied Biosystems. Part of the treatment biological samples receive with this process is to be digested with proteinase K, after which it is permanently inactivated by a suicide

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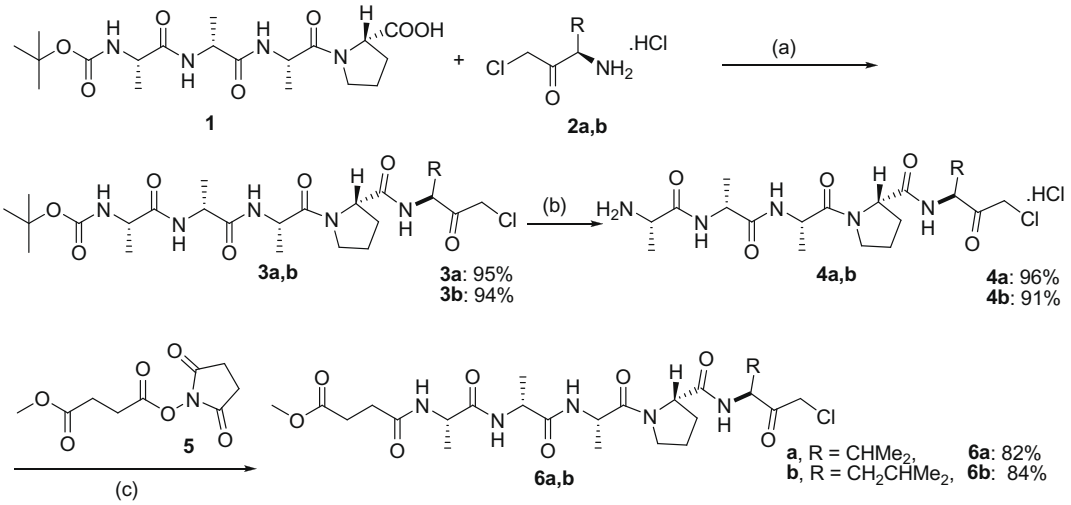

Scheme 1. Reagents and conditions: (a) Isobutyl chloroformate, *N*-methylmorpholine, 4 °C, 15 h; (b) 6 N HCl/EtOAc, rt, 3 h; (c) THF/$NaHCO_3$, rt, 3 h.

inhibitor. To date, there have been no reports that have described a synthesis of modified pentapeptides and validation against proteinase K inhibition activities. In order to investigate the potency of **6a** and **6b** against the proteinase K inhibitor activity, we have used the TaqMan® gene expression Cells-to-CT™ kit and TaqMan® Cells-to-CT™ control kit. (Applied Biosystems), using the new putative inhibitors in place of the current inhibitor, MeOSuc-AAPV-CH_2Cl as a control. The stop solutions with and without **6a** and **6b** inhibitor were prepared and reverse transcription and real time PCR were carried out.[12,13] Reverse transcription was performed using a primer specific for an exogenously-added template, Xeno RNA. The inhibitors were added at various concentration inhibitors prior to addition of the RT reaction components, and the reaction was incubated at 37 °C for 1 h, followed by deactivation of the RT enzyme by incubation at 95 °C for 5 min and cooling the reactions at 4 °C, before adding the PCR components and performing the real-time analysis using the GeneAmp® 9700 PCR system (Applied Biosystems).[13] The PCR components were the TaqMan® Cells-to-CT™ control kit for the Xeno RNA.[14] (Applied Biosystems) The outcome of the analysis of RT and qPCR with new proteinase K inhibitors **6a** and **6b** was compared with the standard inhibitor as a control MeOSuc-AAPV-CH_2Cl, and their average Ct values plotted against the various concentrations of the pentapeptides (Fig. 1).

As shown in Figure 1, the control proteinase K inhibitor MeOSuc-AAPV-CH_2Cl has a plateau of inhibition for proteinase K from 1 mM to 0.25 mM concentration, resulting in a Ct of about 29, while the new pentapeptide inhibitor MeOSuc-AAAPL-CH_2Cl **6b** extends this effective range down to 0.05 mM concentration, again resulting in a Ct of about 29. Clearly, the data indicate that the compound **6b** is fivefold better proteinase K inhibitor than the control. Interestingly, the pentapeptide of MeOSuc-AAAPV-CH_2Cl **6a** requires 0.75 mM concentration to yield 29 Ct. The **6a** did not produce any Ct values below 0.5 mM concentration. The **6b** modified pentapeptide has exhibited the best proteinase K inhibitory activity. This could be due to the formation of a more stable complex

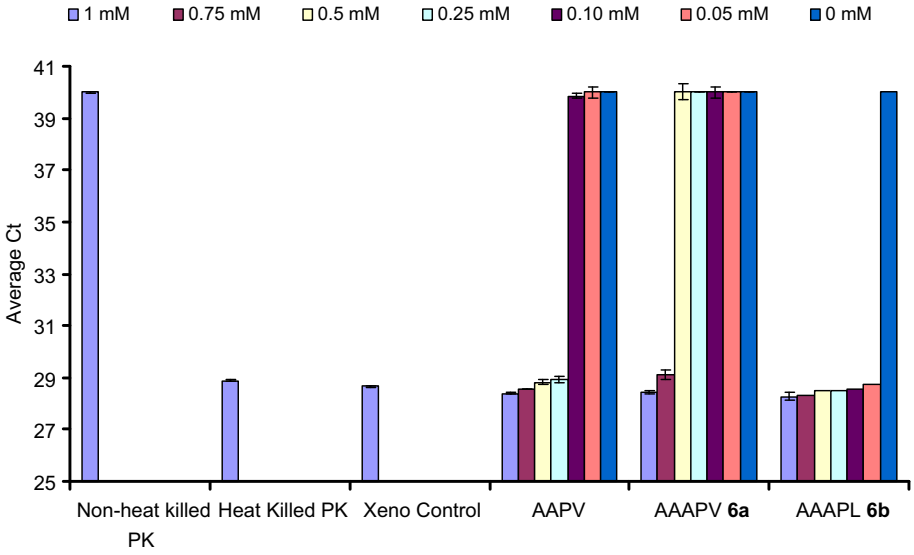

Figure 1. Histograms of average Ct (cycle threshold) values were plotted against the varying concentration of controls MeOSuc-AAPV-CH_2Cl, **6a** and **6b**. The term 'Ct' represents the PCR cycle number when the signal is first recorded as statistically significant. Thus, the lower the Ct value, the greater the concentration of nucleic acid target. Heat killed sample represents the sample containing stop solution which was kept at 95 °C for 10 min in order to fully inactivate any proteinase K activity. Ct values of 40 represents samples with residual proteinase K activity, thereby inhibiting the reverse transcription (RT) reaction. The only control Xeno represents samples in which Xeno RNA was spiked into deionized water.

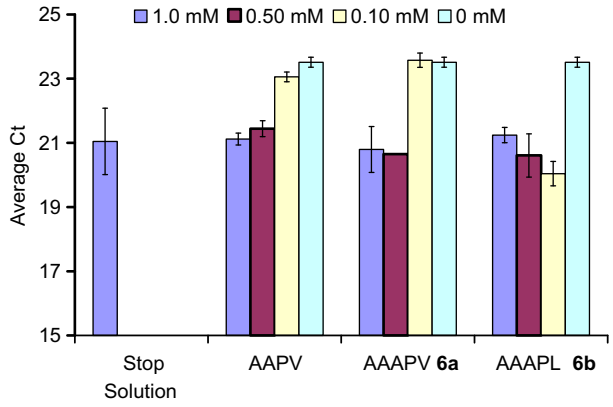

Figure 2. In vitro validation data showing the detection of β-actin in HeLa cell cultures. The control inhibitor MeOSuc-AAPV-CH_2Cl and **6a** inhibits proteinase K at a concentration of 0.5 mM. Similarly, the modified pentapeptide **6b** provides effective inhibition of proteinase K at a concentration of 0.10 mM.

between the leucine residue and proteinase K (binding free energy −30.89 kcal/mol), while **6a** forms a very weak complex between valine and proteinase K (binding free energy 1.32 kcal/mol). The binding free energy of control inhibitor MeOSuc-AAPV-CH_2Cl was −6.63 kcal/mol.

The high proteinase K inhibitory activity of modified pentapeptides are further validated by in vitro detection of β-actin RNA in HeLa Cells (Fig. 2). For this purpose, HeLa cells (~10,000 cells) were lysed in 50 μL lysis solution for 5 min at room temperature. Stop solution (5 μL) was then added; the lysate-stop solution was mixed, and incubated for 2 min at room temperature. A volume of 10 μL of stopped lysate was added to a 50 μL RT reaction followed by addition of 4 μL of RT reaction into a 20 μL PCR reaction using the β-actin primers/probe of the TaqMan® Gene Expression Cells-to-CT™ Kit (Applied Biosystems).

To understand the mechanism of binding of the inhibitors **6a** and **6b** into the binding site of proteinase K and calculate their binding affinity, molecular docking study was conducted using CDOCKER of Discovery Studio V2.01 that uses a CHARMm based Molecular Dynamics docking algorithm.[15–17] The CDOCKER algorithm can generate several different orientations for each inhibitor in proteinase K's active site within the site sphere and perform MD-based simulated annealing followed by final minimization and rank the poses based on CDOCKER energy. The performance of each inhibitor was then re-ranked based on their binding energy. The crystal structure of the inhibitor (MeOSuc-Ala-Ala-Pro-Ala-CH_2Cl)–proteinase K complex was used as a starting structure for molecular simulation (PDB CODE: 3PRK).[8] Water molecules in the protein complex were removed and the resultant structure was energy minimized using CHARMm force field with a convergence threshold of 0.05 kcal/mol Å. The protein part of the complex was used as a receptor and the inhibitor part of the complex was used to build the structures of MeOSuc-AAPV-CH_2Cl, MeOSuc-AAAPV-CH_2Cl **6a** and MeOSuc-AAAPL-CH_2Cl **6b** (Fig. 3). The top 3 docked poses that have less inter-atomic distance (generally less than 3.5 A) between the chloromethyl carbon of the inhibitor and His^{69} (N_ε) of proteinase K were selected and rescored using a physics-based molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) implicit solvation model that ranks the docked inhibitor poses in terms of their binding energy.[16] The RMSA for these three poses were generally less than 0.05 Å. The molecular modeling calculation reveals that pentapeptide **6b** has the lowest binding free energy of −30.89 kcal/mol, followed by control MeOSuc-Ala-Ala-Pro-Val-CH_2Cl with −6.63 kcal/mol and pentapeptide **6a** with 1.32 kcal/mol. The higher binding affinity of **6b** for proteinase K over other two inhibitors MeOSuc-AAPV-CH_2Cl and **6a** as revealed by docking results were corroborated by the higher inhibitory efficiency of MeOSuc-AAAPL-CH_2Cl **6b** compared to inhibitors MeOSuc-AAPV-CH_2Cl and **6a**.

The high proteinase K inhibitory activity of **6b** was further confirmed by an independent assay looking at the digestion of BSA by proteinase K solution in the presence of each of the putative inhibitors. A direct assay for inhibition of proteinase K activity by **6b** was carried out using bovine serum albumin (BSA) as substrate and analyzing products using precast protein gels.[18,19] Such direct assays were carried out to separate inhibition of proteinase K by a test inhibitor from any effects the stopped mixture may have on an RT-PCR reaction.

The outcome of the direct assay (Fig. 4) shows that **6b** was capable of inhibiting proteinase K (100 μg/mL) at concentrations as low as 0.10 mM, while MeOSuc-AAPV-CH_2Cl was only capable of inhibiting proteinase K (100 μg/mL) at a concentrations as low as 0.25 mM.[7] This data demonstrate that the results of the indirect linked assay of MeOSuc-AAPV-CH_2Cl, and **6b** are due to inhibition of the proteinase K by the test inhibitors and are not due to an inhibition of the RT-PCR reaction.

In summary, we have synthesized and evaluated the inhibitory efficiency of two new potent proteinase K inhibitors **6a** and **6b**. Among the two inhibitors, **6b** surpassed the inhibitory efficiency of MeOSuc-AAPV-CH_2Cl, a widely used standard inhibitor for in vitro biological applications, that the former could inhibit proteinase K at fivefold lower concentration than the latter. Also, the inhibitory efficiency of **6b** was much greater than **6a**, enlightening the imporance of the identity of amino acid required at P1 position of the inhibitor for better inhibition. The molecular modeling study also reveals higher binding free energy for the binding of **6b** with proteinase K compared to **6a** and MeOSuc-AAPV-CH_2Cl which is in accordance with their inhibitory efficiencies. Considering the superior inhibitory efficiency of **6b** over the currently used MeOSuc-AAPV-CH_2Cl, one can expect their applications in molecular

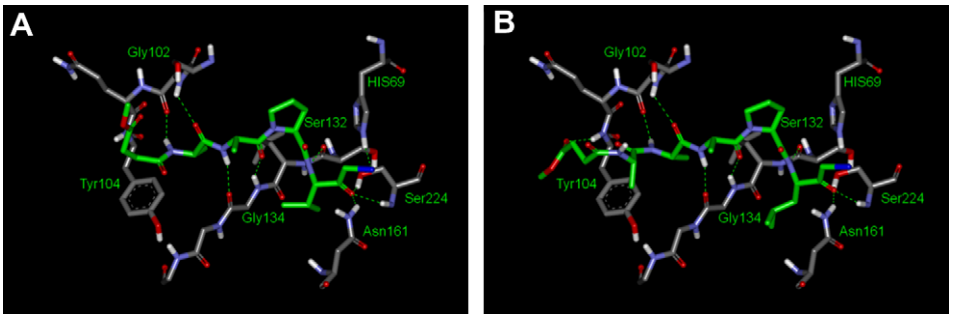

Figure 3. Molecular dynamics based docked proteinase K complexes of MeOSuc-AAPV-CH_2Cl (A) and **6b** (B). The docked complexes were rescored using a physics-based molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) implicit solvation model that ranks the docked inhibitor poses in terms of their binding energy.

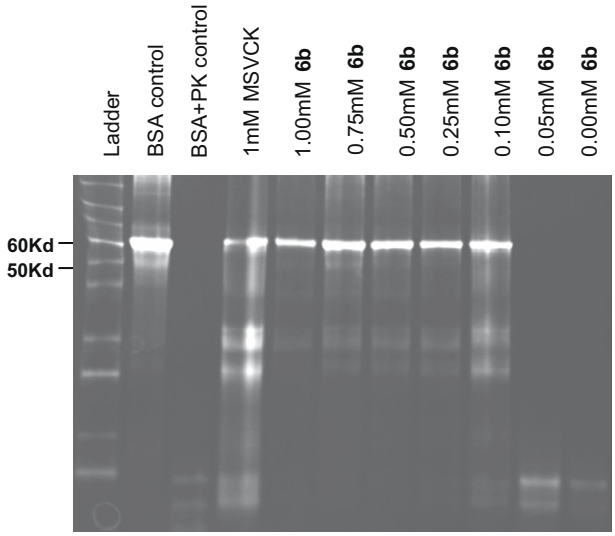

Figure 4. Independent digestion of proteinase K in presence of BSA and the inhibitors MeOSuc-AAPV-CH_2Cl and **6b**.

biology, such as in RT-qPCR, where inhibition of proteinase K is required after their role in disgesting cellular components. Since, proteinase K is inhibited in situ, the removal of proteinase K is not needed and RT-qPCR can be performed in the same reaction mixture eliminating another sample preparation step required otherwise.

Acknowledgments

We are thankful to Helen Chen for assisting in gel assay. We also thank to Debra L. Thompson for encouragement.

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10. *Data for MeOSuc-AAAPV-CH_2Cl* ***6a***: ^{1}H NMR (D_2O, 400 MHz) δ 7.58 (d, J = 8.4 Hz, 1H), 7.52 (d, J = 7.2 Hz, 1H), 6.97 (d, J = 8.0 Hz, 1H), 6.39 (d, J = 6.4 Hz, 1H), 4.59 (m, 3H), 4.54 (m, 1H), 4.41 (m, 1H), 4.36 (d, J = 16.4 Hz, 1H), 4.24 (d, J = 16.4 Hz, 1H), 3.74 (m, 1H), 3.65 (s, 3H), 3.57 (m, 1H), 2.80 (m, 1H), 2.63 (m, 1H), 2.49 (m, 2H), 2.26–1.94 (m, 5H), 1.39 (d, J = 6.8 Hz, 3H), 1.34 (d, J = 6.8 Hz, 3H), 1.32 (d, J = 6.8 Hz, 3H), 0.90 (d, J = 6.8 Hz, 3H), 0.81 (d, J = 8.4 Hz, 3H); MS (m/z): 574 $[M+H]^+$.
11. *Data for MeOSuc-AAAPL-CH_2Cl* ***6b***: ^{1}H NMR (D_2O, 400 MHz) δ 7.44 (d, J = 7.6 Hz, 1H), 7.42 (d, J = 7.2 Hz, 1H), 7.02 (d, J = 8.4 Hz, 1H), 6.32 (d, J = 6.0 Hz, 1H), 4.55 (m, 4H), 4.40 (d, J = 16.4 Hz, 1H), 4.36 (m, 1H), 4.26 (d, J = 16.4 Hz, 1H), 3.68 (m, 1H), 3.64 (s, 3H), 3.55 (m, 1H), 2.83 (m, 1H), 2.62 (m, 1H), 2.47 (m, 2H), 2.12–1.90 (m, 5H), 1.58 (m, 2H), 1.41 (d, J = 6.8 Hz, 3H), 1.35 (d, J = 8.0 Hz, 6H), 0.90 (d, J = 6.0 Hz, 3H), 0.85 (d, J = 6.4 Hz, 3H); MS (m/z): 588 $[M+H]^+$.
12. *Preparation of stop solution with inhibitor*: Typically, stop solution was prepared in 100 μL volumes, containing the following components: Tris pH 8.0, 11 mM; EGTA pH 9.0, 88 mM; MeoSuc-AAPV-CH_2Cl, **6a**, and **6b** separately in each tube at final concentration of 11 mM; placental RNase inhibitor protein, 2.2 U/μL; and DTT, 0.11 mM. The solution pH was adjusted to 8.0, if required, by dilute HCL. A dilution solution was prepared identically, except that inhibitor analog was omitted. A series of solutions was made for each inhibitor were made by diluting the initial solutions with the dilution solution so that the final concentrations were 1 mM (100%), 0.75 mM (75%), 0.5 mM (50%), 0.25 mM (25%), 0.10 mM (10%), 0.05 mM (5%) and control 0 m mM (0%).
13. *Reverse transcription reactions*: All reactions were carried out in triplicate. Fifty microliters of proteinase K solution (without DNase mixed in) from the TaqMan® gene expression Cells-to-CT™ kit (Applied Biosystems) were aliquoted into 18 tubes. To each of these (these are equivalent to lysis mixtures in the Cells-to-CT™ workflow), 5 μL of the various stop solutions of MeoSuc-AAPV-CH_2Cl, **6a**, and **6b** were added separately in each tube, resulting in 1 mM, 0.75 mM, 0.5 mM, 0.25 mM, 0.10 mM, 0.05 mM and control 0 m mM concentrations of the inhibitor analogs in the proteinase K solution. These were incubated 2 min at room temperature. For the control reaction, 50 μL of proteinase K solution was mixed with 5 μL of the dilution solution and heated at 95 °C for 10 min to thermally inactivate the proteinase K reaction. Next, the reverse transcription reagents were prepared, that is, 15 μL 2× RT buffer, and 1.5 μL of 20× RT enzyme mix, were aliquoted and mixed in the wells of a 96 well plate. To this solution, 13.5 μL of each proteinase K + stop mixture was added and the solution was mixed well. The resulting mixtures, containing various concentration of the appropriate analog was incubated at room temperature for 1 h for residual PK activity to degrade the RT enzyme. After 1 h incubation at room temperature, 1 μL of Xeno RNA (diluted to 25,000 copies/μL in 10 ng/μL poly(A) was added to all wells except for the no template control wells. Then the reverse transcription (RT) reaction was incubated at 37 °C for 60 min, 95 °C for 5 min, and cooled to 4 °C, using the GeneAmp® 9700 PCR system (Applied Biosystems).
14. *Real time PCR.* Real time PCR was performed by using the Xeno AOD (Assay on Demand) TaqMan® Cells-to-CT™ control kit, on a 7500 Fast Real-Time PCR system. (Applied Biosystems) In each reaction, the equivalent of 12.5 μL of TaqMan GEX master mix mixed with 1.25 μL of 20× Xeno AOD was dispensed in each well of a 96 well plate. 11.25 μL of each RT reaction was then added to each well. For three control reactions, 11.25 μL of water was added to the three wells. Then the 96 well plate was run by using the gene expression parameters as per manufacturer's instruction.
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17. *Docking study of proteinase k inhibitors into the binding site of proteinase K.* To understand the mechanism of binding of the inhibitors MeOSuc-AAPV-CH_2Cl, MeOSuc-AAAPV-CH_2Cl **6a**, and MeOSuc-AAAPL-CH_2Cl **6b** into the binding site of proteinase K and calculate their binding affinity, molecular docking study was conducted using CDOCKER of Discovery Studio V2.01 that uses a CHARMm based Molecular Dynamics docking algorithm. The CDOCKER algorithm can generate several different orientations for each inhibitor in proteinase K's active site within the site sphere and perform MD-based simulated annealing followed by final minimization and rank the poses based on CDOCKER energy. The performance of each inhibitor was then re-ranked based on their binding energy. The crystal structure of the inhibitor–proteinase K complex was used as a starting structure for molecular simulation (PDB CODE: 3PRK). Water molecules in the protein complex were removed and the resultant structure was energy minimized using CHARMm force field with a convergence threshold of 0.05 kcal/mol Å. The protein part of the complex was used as a receptor and the inhibitor part of the complex was used to build the structures of MeOSuc-AAPV-CH_2Cl, MeOSuc-AAAPV-CH_2Cl **6a** and MeOSuc-AAAPL-CH_2Cl **6b**. The top 3 docked poses that have less inter-atomic distance (generally less than 3.5 Å) between the chloromethyl carbon of the inhibitor and $His^{69}(N_\varepsilon)$ of proteinase K were selected and rescored using a physics-based molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) implicit solvation model that ranks the docked inhibitor poses in terms of their binding energy. The RMSD for these three poses were generally less than 0.05 Å. The total binding free energy of the inhibitors binding into the active site of proteinase K was calculated using the following Eq. 1.

$$\Delta\Delta G_{\text{Bind}} = \Delta G_{\text{Complex}} - \Delta G_{\text{Ligand}} - \Delta G_{\text{Protein}} \quad (1)$$

The docking study reveals several interesting features of which three are worth mentioning. First, the interaction pattern of docked inhibitors MeOSuc-AAPV-CH_2Cl, **6a** and **6b** were similar to the one that is observed with the original proteinase K-MeOSuc-AAPA inhibitor structure (PDB: 3PRK) that include the bifurcated hydrogen bonds observed between Ser^{224} and Asn^{161} of proteinase K with the carbonyl of valine/leucine residue of the docked inhibitor. Second, MeOSuc-AAPV-CH_2Cl forms three more hydrogen bonds with the proteinase K compared to MeOSuc-AAAPV-CH_2Cl **6a**. There were nine intermolecular hydrogen bonds observed between proteinase K and MeOSuc-AAPV-CH_2Cl. Third, MeOSuc-AAAPL-CH_2Cl **6b** was exhibiting lowest binding free energy of -30.89 kcal/mol compared to MeOSuc-AAPV-CH_2Cl and MeOSuc-AAAPV-CH_2Cl whose binding energies were −6.63 kcal/mol and 1.32 kcal/mol, respectively. The higher binding affinity of MeOSuc-AAAPL-CH_2Cl **6b** for proteinase K over other two inhibitors MeOSuc-AAPV-CH_2Cl and **6a** as revealed by docking results were corroborated by the higher inhibitory efficiency of MeOSuc-AAAPL-CH_2Cl **6b** compared to inhibitors MeOSuc-AAPV-CH_2Cl and **6a**.
18. *Independent digestion assay of MeOSuc-AAPV-CH_2Cl and* ***6b***: For the direct assay of PK inactivation, 50 μL of proteinase K (100 μg/mL) solution was mixed with 5 μL of stop solution with varying amounts of MeOSuc-AAPV-CH_2Cl and **6b** separately and incubated for 10 min at room temperature. Ten microliters of Ultrapure BSA (5 mg/mL) were added to each sample and the samples were incubated for 10 min at room temperature. The reaction mixture was heated

for 30 min at 95 °C to inactivate any remaining functional PK and the sample mixes analyzed using precast protein gels. For each 65 μL of total reaction mixture, 10 μL was mixed with 5 μL of gel loading dye and further heated at 95 °C for 5 min. Then, the samples were kept on ice for 2 min and 15 μL of reaction mixture was loaded on the gel along with ladder. For the control reactions, 50 μL of PK solution without BSA, and 10 μL of BSA were mock-incubates and analyzed in a similar fashion.

19. *Protein gel*: The precast protein gel was run at 120 V for 1 h using 1× Tris/glycine/SDS buffer (BIO-RAD, Hercules, CA). The gel was stained with Coomassie blue stain for 1 h at room temp and destained with destaining solution (20% acetic acid, 10% methanol in 1 L). The gel was analyzed using AlphaEase™ FC software (Alpha Innotech, San Leandro, CA). The protein ladder ranges in size from 10 kDa to 260 kDa (Novex® Sharp Pre-Stained Protein Standard (Invitrogen, Carlsbad CA).