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Biochemical and Docking Analysis of Substrate Interactions with Polyisoprenylated Methylated Protein Methyl Esterase

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Abstract

Polyisoprenylated proteins (PPs) methylation by polyisoprenylated protein methyl transferase (PPMTase) is counteracted by polyisoprenylated methylated protein methyl esterase (PMPMEase). This is the only reversible step of the polyisoprenylation pathway as the relative amounts of the acid and ester forms are determined by the two competing reactions. Since PMPMEase and PPMTase may influence both the structural/functional conformations of PPs, a thorough study of these enzymes is essential to our understanding of the structural/functional features of PPs. PMPMEase has been reported under such pseudonyms as human carboxylesterase 1 (hCE1) because of its apparent broad substrate spectrum. The current study aimed to show the complementarity between its active site and the polyisoprenylated substrates that it metabolizes. Kinetics analysis was conducted with N-, S- and O-substituted substrates using porcine liver PMPMEase and docking analysis using Arguslab. Consistent with the biochemical analysis, the S-ethyl analog yielded an AScore binding energy of -11.32 compared to -13.48, -14.88, -16.15, and -16.81 kcal/mol for S-prenyl (C-5), S-*trans*-geranyl (C-10), S-*trans,trans*-farnesyl (C-15) and S-*all trans*-geranylgeranyl (C-20), respectively. The *all trans*-geranylgeranyl moiety provides the optimal size for active site interactions. The data reveal that the *trans,trans*-farnesyl and *all trans*-geranylgeranyl groups, which are reminiscent of endogenous PPs modifications, have the highest affinity for PMPMEase. Since PPs such as monomeric G-proteins are oncogenic, PMPMEase may be viewed as a viable target for anticancer drug development. The analyses reveal the important structural elements for the design of specific PMPMEase inhibitors to serve in the modulation of oncogenic PPs activities. The results also show that hCE1's repertoire of substrates extends beyond xenobiotics to include PPs as its endogenous substrates.

Introduction

The polyisoprenylation pathway (PP) involves three sets of sequential modifications initiated by the farnesylation or geranylgeranylation of the cysteine residue of the CaaX signal motif [1]. This posttranslational modification is initiated by either a farnesyl or a geranylgeranyl transferase [2-5]. An endoprotease then cleaves the aaX tripeptide, exposing the resulting carboxylate of the polyisoprenylated cysteine to methylation by polyisoprenylated protein methyl transferase (PPMTase). The methyl esterified polyisoprenylated proteins are the substrates of a polyisoprenylated methylated protein methyl esterase (PMPMEase) that in conjunction with PPMTase render the methylation the only reversible step of the PP [6-9].

Mutations and overexpression that result in hyperactivity of polyisoprenylated monomeric G-proteins are implicated in about 30% of cancers [10] while impaired activities of some PP enzymes are linked to degenerative disorders [11,12]. The hyperactivity of the oncogenic PPs stems from mutations that destroy the autoregulatory GTPase activities of PPs such as members of the Ras family [13,14]. Overexpression of Rho family proteins also causes hyperactivity in some cancers [15,16]. Realizing that the PP modifications are essential for the functions of the oncogenic PPs, key enzymes of the PP have been targeted for anti-cancer drug development. Development of farnesylation inhibitors has been the main focus of the effort to obtain clinically useful PPs-dependent anticancer agents [17-21]. Compounds from this category have reached various phases of clinical trials [22,23]. The main impediment to their success has been the ability of geranylgeranyl transferase to substitute for the inhibited farnesyl transferase, ultimately compromising the antiproliferative effects of the farnesyl transferase inhibitors [24,25]. The lethality observed for PPMTase knockout mice [6] has also drawn attention to its physiological relevance and its significance as a target for anticancer drug development [10,26].

Research in our lab has focused on the identification [26,27] and substrate characterization of PMPMEase [28]. Porcine liver PMPMEase is 79% identical and 88% similar to human carboxylesterase 1 (hCE1). hCE1 is a well characterized enzyme whose crystal structure in complex with various prodrug and xenobiotic molecules have been solved [29,30]. The crystallographic data shows its active site to be large, hydrophobic and flexible [30,31]. The hydrophobic nature is most likely an adaptation for interacting with the hydrophobic polyisoprenyl groups of PPs while the size and flexibility of the active site may be required to interact with the polypeptides that vary enormously in type, size and structure [30].

Previous work in our laboratory [28] used Michaelis-Menten kinetics to show a strong dependence of porcine PMPMEase on *S-trans*, *trans*-farnesylation and *all trans*-geranylgeranylation for high affinity interactions. In the current article, the same approach was used to further characterize the nature of the active site. In addition, Arguslab version 4.0.1 (Planaria Software LLC, Seattle, WA) [31] docking software was used to determine the relative binding energies of the substrates to two hCE1 crystal structures [35,36], the rabbit homolog [37] and the model of porcine liver enzyme. The docking analyses reveal the *S-trans*, *trans*-farnesyl and *all trans*-geranylgeranyl moieties of the substrate closely interacting with hydrophobic amino acid residues in the active site and the susceptible ester bond positioned adjacent to the catalytic Ser221. The relative location of the N-4-nitrobenzoyl group at the exit of the active site is noteworthy. Given that the N-4-nitrobenzoyl group occupies the analogous position of the polypeptide chains in PPs, this finding is consistent with our expectations as these polypeptide portions should extend to the outside of the enzyme active site during metabolism.

Materials and Methods

Materials

Reagents were purchased either from Sigma-Aldrich (Milwaukee, WI), Acros (Morris Plains, NJ), TCI America (Portland, OR), Alfa Aesar (Ward Hill, MA) or ChemImpex International (Wood Dale, IL) and used as supplied. Flash column chromatography was run on 40-63 μ SiO₂. NMR spectra were obtained on a Varian Mercury 300 (300 MHz) in CDCl₃, unless otherwise stated. Chemical shifts (δ) are given in ppm relative to the signal for the deuterated solvent and are reported consecutively as relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet and where br = broad), coupling constant (J/Hz) and assignment. IR spectra were measured using Shimadzu 840S in KBr pellets. Optical rotations were measured with a JASCO P-1020 polarimeter in a 10cm cell. Elemental analysis was conducted by Atlantic Microlab,

Inc, Norcross, GA, and are within 0.4% of the theoretical values except otherwise noted. ArgusLab 4.0.1 was obtained from Mark A. Thompson (Planaria Software LLC, Seattle, WA, www.arguslab.com).

Synthesis of N-derivatized S-farnesyl-L-cysteine methyl ester substrates

All synthetic experiments were conducted under argon in flame-dried glassware, when required. N-substituted S-farnesyl-L-cysteine methyl esters were synthesized essentially as previously described ([32] Scheme 1). *trans,trans*-farnesyl-L-cysteine methyl ester (FCM) was synthesized as previously described [26-28].

Synthesis of L-N-(1-naphthoic)-S-farnesyl-cysteine-O-methyl ester (L-150)

N,N Dicyclohexyl-carbodiimide (DCC) in dichloromethane (DCM) (0.7 mL, 0.7 mmol) was added to a stirred suspension of 1-naphthoic acid (103mg, 0.60mmol) and FCM (204mg, 0.60mmol) in anhydrous DCM (5 mL). The mixture was kept at 20 °C for one day. Urea was filtered out of the solution and solvent removed under vacuum (3 mmHg) at 35°C over 20 min. The solid residue was dissolved and purified on a 40-63 μ silica gel flash column (2.2x8 cm) eluting with a stepwise gradient of hexane- ethylacetate of 8:1 (100 mL) and 5:1 (100 mL). The solvent was removed under vacuum to obtain the yellowish oil (251mg, 54%). ¹H-NMR: δ 1.42 (s, CH₃), 1.42 (s, CH₃), 1.66 (s, CH₃), 1.95-2.09 (m, 4 $\times$ CH₂), 3.02-3.11 (β' and β) (dd, S-C-H J =7.5Hz), 3.012-3.24 (t, CH₂S, J =7.2Hz), 3.81 (s, OCH₃), 4.97-5.04 (d, HC-NH₂), 5.04-5.08 (broad, 2 $\times$ C=CH), 5.21-5.22 (broad, C=CHCS, J =1.2Hz), 7.52-7.56 (d, Ar), 7.84-7.93 (d, Ar), 8.35 (d, HN, J =10.2Hz). ¹³C-NMR: δ 16.27, 16.38, 17.82, 25.89, 26.64, 26.95, 30.38, 33.76, 39.83, 39.91, 52.60, 52.91, 119.8, 123.8, 124.0, 124.6, 127.01, 128.0, 128.7, 129.25, 131.2, 131.5, 132.8, 135.2, 140.4, 167.3, 171.8. Calcd for C₃₀H₃₉NO₃S: C, 72.98; H, 7.96; N, 2.84. Found: C, 72.89; H, 8.01; N, 2.82.

Synthesis of L-N-(2-Naphthoic)-S-*trans,trans*-farnesyl-cysteine methyl ester (L-151)

Triethylamine (TEA, 181 μ L, 1.32 mmol) was added to a stirred suspension of 2-Naphthoic acid (114 mg, 0.6 mmol) and FCM (204 mg, 0.6 mmol) in anhydrous DCM (10 mL) on ice. After 10 min, the mixture was left at RT for 4 h. The solvent was removed under vacuum and the solid residue dissolved in ethylacetate. The organic phase was washed with cold 5% NaHCO₃, 10% HCl and brine, dried over MgSO₄ and the solvent removed under vacuum. The resulting oil was purified by flash chromatography on a silica gel column eluting with DCM to afford a solid (108mg, 36.5%). ¹H-NMR: δ 1.42 (s, CH₃), 1.58 (s, CH₃), 1.66 (s, CH₃), 1.96-2.10 (m, 4 $\times$ CH₂), 3.02-3.11 (β' and β) (dd, S-C-H J =7.5Hz), 3.012-3.24 (t, CH₂S, J =7.2Hz), 3.81 (s, OCH₃), 4.97-5.04 (d, HC-NH₂), 5.04-5.08 (br, 2 $\times$ C=CH), 5.21-5.22 (br, C=CHCS), 7.32-7.46 (d, Ar), 7.62-7.70 (d, Ar), 7.79-7.82 (d, Ar), 7.84-7.93 (d, Ar), 8.35 (d, HN, J =10.2Hz). ¹³C-NMR: δ 16.27, 16.38, 17.82, 19.6, 25.89, 26.64, 26.95, 30.38, 33.76, 39.83, 39.91, 52.60, 52.91, 119.8, 123.8, 124.0, 124.6, 127.01, 128.0, 128.7, 129.25, 131.2, 131.5, 132.8, 135.2, 140.4, 167.3, 171.8. Calcd for C₃₀H₃₉NO₃S: C, 72.98; H, 7.96; N, 2.84. Found: C, 72.89; H, 8.02; N, 2.79.

L-N-(4-Phenylbenzoyl)-S-*trans,trans*-farnesyl-cysteine methyl ester (L-152)

A 1 N solution of DCC in DCM (1.5 mL, 1.5 mmol) was added to a stirred suspension of 4-phenylbenzoic acid (119 mg, 0.6 mmol) and FCM (204 mg, 0.6 mmol) in anhydrous DCM (10 mL) and the mixture was kept at RT for one day. Dicyclohexylurea (DCU) was filtered out and the solvent removed under vacuum. The residue was dissolved in DCM and separated on a flash column on a stepwise gradient of Hexane- ethylacetate 10:1 to 2:1. Crystallization from methanol with activated carbon gave yellow solid (30mg, 9.6 %); mp 77-79°C; $[\alpha]_D^{20}$ = -27.78. ¹H-NMR: δ 1.55-1.60 (s, CH₃), 1.61-1.66 (s, CH₃), 1.59 (s, CH₃), 1.66 (s, CH₃), 1.93-2.10 (m, 4 $\times$ CH₂), 2.82-2.90 (H β) and 2.95-3.01 (H β') (dd, S-C-H

$J=7.5\text{Hz}$), 3.01-3.24 (t, CH_2S , $J=7.2\text{Hz}$), 3.77 (s, OCH_3), 4.276 (d, 2 $\times$), 4.78-4.84 (t, HC-NH_2), 5.05-5.09 (m, 2 $\times$ C=CH), 5.15-5.20 (m, C=CHCS , $J=1.2\text{Hz}$), 7.21-7.27 (d, HN, $J=10.2\text{Hz}$), 7.58 (s, NH), 7.98 (m, Ar, 2H, 2H), 8.27 (m, Ar, 2H, 2H). $^{13}\text{C-NMR}$: δ 16.27, 16.39, 17.95, 25.97, 26.64, 26.93, 30.07, 33.28, 39.87, 39.27, 43.71, 52.25, 53.08, 119.6, 123.8, 124.0, 124.5, 128.7, 131.6, 135.7, 139.2, 140.5, 149.9, 165.8, 168.9, 171.3. Calcd for $\text{C}_{32}\text{H}_{41}\text{NO}_3\text{S}$: C, 73.95; H, 7.95; N, 2.69. Found: C, 73.67; H, 8.00; N, 2.69.

L- N-(3, 5 Dinitrobenzoic)-S-farnesyl-cysteine-O-methyl ester (L-155)

DCC in DCM (0.7mL, 0.7mmole) was added to a stirred suspension of 3,5 dinitrobenzoic acid (191mg, 0.90mmol) and FCM (306 mg, 0.90mmol) in anhydrous DCM (5mL). The mixture was kept at 20 °C for one day. Urea was filtered out of the solution and the solvent removed under vacuum (3mmHg) at 35°C. The solid residue was dissolved in a hexane-ethylacetate (8:1) mixture and applied on a 40-63 μ silica gel flash-column 2.2 $\times$ 8 cm, eluting with a stepwise gradient of hexane- ethylacetate from 8:1 (100 mL) to 5:1 (100 mL). The solvent was removed and the product dried under vacuum yielding yellow oil (251 mg, 54%). $[\alpha]_D^{24}=-63.7$. $^1\text{H-NMR}$: δ 1.57 (s, CH_3), 1.63 (s, CH_3), 1.66 (s, CH_3), 1.94-2.10 (m, 4 $\times$ CH_2), 3.08-3.10(β' and β) (d, S-C-H $J=7.5\text{Hz}$), 3.19-3.21 (d, CH_2S , $J=7.2\text{Hz}$), 3.81 (s, OCH_3), 4.52-4.55 (d, HC-NH_2), 5.03-5.07 (br, 2 $\times$ C=CH), 5.20-5.21 (br, C=CHCS , $J=1.2\text{Hz}$), 7.86-7.890 (d, HN, $J=10.2\text{Hz}$), 8.26 (d, Ar), 9.10-9.12 (d, Ar $\text{O}_2\text{N-CH-NO}_2$). $^{13}\text{C-NMR}$: δ 16.2, 16.4, 17.9, 25.9, 26.6, 26.9, 30.5, 33.5, 39.8, 39.9, 53.4, 56.2, 114.4, 119.5, 123.8, 124.4, 124.5, 130.5, 131.5, 131.5, 135.7, 137.1, 140.8, 147.3, 163.0, 170.2. Calcd for $\text{C}_{26}\text{H}_{35}\text{N}_3\text{O}_7\text{S}$: C, 58.52; H, 6.61; N, 7.87; Found: C, 59.01; H, 6.62; N, 7.87.

L-N-(3-nitrobenzoyl)-S-trans,trans-farnesyl-cysteine methyl ester (L-156)

TEA (181 μL , 1.32 mmol) was added to a stirred suspension of FCM (393 mg, 1.15 mmol) in anhydrous DCM (10mL) on ice. 3-Nitrobenzoyl chloride (273 mg, 1.47 mmol) in DCM was added drop-wise to the reaction mixture. After 60min on ice, the mixture was left at RT for 4 h. The solvent was removed under vacuum and the solid residue dissolved in ethyl acetate, washed with cold 5% NaHCO_3 , 10% HCl and brine and dried over MgSO_4 . The solvent was evaporated and the resulting oil purified by flash chromatography on a silica gel column eluting with hexane- ethylacetate (3:1) to give a solid (440.4mg, 77%). $^1\text{H-NMR}$: δ 1.58 (s, CH_3), 1.59 (s, CH_3), 1.66 (s, CH_3), 1.94-2.10 (m, 4 $\times$ CH_2), 2.98-3.09 (β' and β) (dd, S-C-H $J=8.1\text{Hz}$), 3.11-3.66 (t, CH_2S , $J=7.2\text{Hz}$), 3.82 (s, OCH_3), 4.97-5.03 (d, HC-NH_2), 5.05-5.08 (br, 2 $\times$ C=CH), 5.10-5.24 (br, C=CHCS , $J=1.5\text{Hz}$), 7.01-7.26 (d, HN, $J=7.5\text{Hz}$), 7.64-7.69 (t, Ar), 8.15-8.19 (d, Ar), 8.37-8.41, (d, Ar). 8.66-8.67 (d, Ar). $^{13}\text{C-NMR}$: δ 16.38, 16.38, 17.82, 25.9, 26.9, 30.3, 33.6, 39.8, 39.9, 52.6, 53.1, 119.6, 122.5, 123.86, 124.2, 126.6, 130.1, 131.1, 133.2, 135.5, 140.1, 145.1, 167.6, 171.1. Calcd for $\text{C}_{26}\text{H}_{36}\text{N}_2\text{O}_5\text{S}$: C, 63.91; H, 7.43; N, 5.73. Found: C, 63.72; H, 7.26; N, 5.64.

L-N-(2-nitrobenzoyl)-S-trans,trans-farnesyl-cysteine methyl ester (L-157)

TEA (181 μL , 1.32 mmol) was added to a stirred suspension of FCM (427.8 mg, 1.47 mmol) in anhydrous DCM (10 mL) on ice. 2-Nitrobenzoyl chloride (273 mg, 1.47 mmol) in DCM was added drop-wise to the reaction mixture. After 60 min on ice, the reaction mixture was left at RT for 4 h. The solvent was removed under vacuum and the solid residue dissolved in ethyl acetate and washed with cold 5% NaHCO_3 , 10% HCl and brine and dried over MgSO_4 . The solvent was evaporated and the resulting oil purified by flash chromatography on a silica gel column, eluting with DCM to give a solid (313mg, 43.4%). $^1\text{H-NMR}$: δ 1.60 (s, CH_3), 1.68 (s, CH_3), 1.72 (s, CH_3), 1.95-2.10 (m, 4 $\times$ CH_2), 2.99-3.06 (β' and β) (dd, S-C-H $J=5.7\text{Hz}$), 3.10-3.29 (t, CH_2S , $J=13.8\text{Hz}$), 3.82 (s, OCH_3), 4.99-5.06 (d, HC-NH), 5.07-5.11 (br, 2 $\times$ C=CH), 5.20-5.25 (br, C=CHCS , $J=5.4\text{Hz}$), 6.64-6.66 (d, HN, $J=7.8\text{Hz}$), 7.26-7.62 (t, Ar), 7.66-7.69 (t, Ar), 8.06-8.09, (d, Ar). $^{13}\text{C-NMR}$: δ 16.38, 16.38, 17.82, 25.9, 26.9, 30.3, 33.6, 39.8, 39.9, 52.6, 53.1, 119.6, 122.5, 123.86, 124.2, 126.6, 130.1,

131.1, 133.2, 135.5, 140.1, 145.1, 167.6, 171.1. Calcd for $C_{26}H_{36}N_2O_5S$: C, 63.91; H, 7.43; N, 5.73. Found: C, 64.16; H, 7.52; N, 5.78.

L- N-(2-(methoxycarbonyl) benzyl)-S-farnesyl-cysteine-O-methyl ester (L-158)

DCC in DCM (0.7mL, 0.7mmol) was added to a stirred suspension of 2-(methoxycarbonyl) benzoic acid (109mg, 0.60mmol) and FCM (204mg, 0.60mmol) in anhydrous DCM (5 mL). The mixture was kept at 20 °C for one day. Urea was filtered out of the solution and the solvent removed under reduced pressure (3 mmHg) at 35°C for 20 min. The solid residue was dissolved in hexane- ethylacetate (10:1) mixture and purified on 40-63 μ flash silica gel column (2.2×8 cm) eluting with a stepwise gradient of hexane- ethylacetate 10:1 (90 mL) to 2:1 (100 mL). The solvent was removed under vacuum to obtain a white solid (150mg, 49.8%). $[\alpha]_D^{24} = -36.92$. 1H -NMR: δ 1.58 (s, CH_3), 1.67 (s, CH_3), 1.67 (s, CH_3), 1.97-2.10 (m, $4 \times CH_2$), 2.96-3.00 (β' and β) (d, S-C-H $J=7.5$ Hz), 3.10-3.23 (d, CH_2S , $J=7.2$ Hz), 3.79 (s, OCH_3), 3.86 (s, OCH_3), 4.99-5.05 (d, $HC-NH_2$), 5.06-5.09 (br, $2 \times C=CH$), 5.10-5.23 (br, $C=CHCS$, $J=1.2$ Hz), 7.46-7.51 (d, HN, $J=10.2$ Hz), 7.52-7.54 (d, Ar), 7.85-7.88 (d, Ar). ^{13}C -NMR: δ 16.2, 16.3, 17.9, 25.9, 26.6, 26.9, 30.3, 33.7, 39.8, 39.9, 52.5, 52.67, 52.76, 119.9, 123.9, 124.5, 127.9, 129.6, 130.0, 130.3, 131.4, 132.1, 135.5, 137.7, 140.2, 167.1, 169.0, 171.4. Calcd for $C_{28}H_{39}NO_5S$: C, 67.03; H, 7.84; N, 2.79. Found: C, 66.80; H, 7.72; N, 2.83.

Enzyme Kinetics Analysis

The putative substrates were screened for hydrolysis by PMPMEase as previously described [27-29]. Hydrolyzed compounds (substrates) were subjected to Michaelis-Menten kinetics analysis as described previously [28]. Varying concentrations of each substrate (3-1000 μ M) were incubated with PMPMEase (0.1 to 5 μ g) in 100 mM Tris-HCl, pH 7.4 containing 5% DMSO at 37°C in a total incubation volume of 100 μ L. Reactions were stopped by adding 200 μ L of methanol. They were then placed on ice for at least 5 min before centrifugation at $5000 \times g$ for 5 min. The supernatants were removed and analyzed by RP-HPLC with UV detection at 260 nm as previously described [27-29]. The product peak areas were measured and used to quantify the amount of product formed using a calibration plot of known amounts of product against peak area. All experiments were conducted in triplicates.

Porcine Liver Esterase Modeling

The crystal structure of porcine liver PMPMEase used in the biochemical analysis has not been solved. On the other hand, hCE1 shares 79% sequence identity and 88% sequence similarity to porcine liver PMPMEase [27]. It is thus believed to be the human version of porcine liver enzyme. Therefore, in the absence of a crystal structure for the porcine enzyme, those for hCE1 with various bound hydrophobic ligands [29,30,33] were considered for docking studies. The structures reveal an active site that is large, flexible and hydrophobic [31,34]. These have been suggested to be the essential elements for binding the polyisoprenes as well as for accommodating the large, structurally varied polypeptides [27]. Furthermore, one of the crystal structures of hCE1 (PDB: 1YAH [33]) was used as a template to construct a structural model for the porcine liver enzyme. To achieve this, the amino acid sequence of porcine liver esterase (Accession: **Q29550**) was retrieved from the SWISS PROT database and aligned with the sequence of hCE1 used for PDB: 1YAH. The porcine PMPMEase model structure (PLE) was then constructed using the MODELLER program [34].

Docking of N, S, O-substituted cysteine methyl ester analogs to the active site of various PMPMEase

Crystallized human carboxylesterase (1YAH) has an ethyl acetate, an analog of fatty acid ethyl ester, bound to the active site [34]. The ethyl acetate was used to create a ligand-binding site for the substrates. This includes various amino acids that lie within a defined pocket. The active site residues were used to create the binding sites for the other human (1MX5) [35,36], porcine (PLE) and rabbit (1K4Y)[37] PMPMEase isoforms. ArgusLab Version 4.01 software was used for the docking analyses. Procedures for the docking of substrates were essentially as previously described [38]. Briefly, a Chemdraw structure for each compound was first saved as an MDL Molfile and opened in ArgusLab. The atoms were then set to the appropriate hybridizations (sp³, sp², etc) followed by the addition of the hydrogen atoms. The geometry of each compound was optimized before it was made into a ligand using the “Make a Ligand Group from this Residue” option of the ArgusLab software. The docking between the binding site and each substrate was performed using the “Dock a ligand” option. A maximum number of 500 poses was set in order to increase the binding precision. All the amino acid residues of the ethyl acetate binding site were defined to be part of the binding site using a cubic box measuring 18 × 18 × 22 points built to include the entire active site. All other docking analysis parameters were set as previously described [38]. Spacing between the grid points was set at 0.4 Å. “ArgusDock” and “Dock” were chosen as the docking engine for the simulations and calculation type, respectively. “Flexible” was chosen for the ligand and “AScore” for the scoring function. The binding energies for the ligands (substrates) were calculated with parameters from the AScore.prm file using the AScore function. Ligands that were previously defined, from ligand setup, were then docked and the AScore energies recorded. Poses were rank-ordered by docking energy and the pose with the lowest energy was chosen as the predicted receptor-bound conformation of the ligand. These poses were examined to confirm the position of the susceptible ester bond relative to the catalytic Ser 221 residue. Molecular viewing was conducted with Visual Molecular Dynamics (VMD) software [39].

Results

Synthesis of N-substituted S-Farnesylated Cysteine Methyl Ester Analogs

Substrates were synthesized according to scheme 1. Once purified, each compound was analyzed by thin layer chromatography, proton and carbon-13 NMR and elemental analysis. The melting points for solid compounds and optical rotations for chiral compounds were determined. The yields ranged from 30 to 60% and the purity of the compounds was close to 99% as determined by NMR, elemental analysis and HPLC.

Michaelis-Menten kinetics analysis of N-substituted S-farnesylated cysteine methyl ester analogs

When the synthesized compounds were initially screened for hydrolysis by PMPMEase, all the compounds except L-152 with the 4-phenyl benzoic acid group were hydrolyzed. The Michaelis-Menten kinetics data show significant differences with respect to the nature of the N-substitutions (Fig. 1, Table 1). L-157 with an N-2-nitrobenzoyl substituent had an approximately 2-fold lower K_M (14 μ M) than the N-3-nitrobenzoyl (L-156, 36 μ M) and the N-4-nitrobenzoyl (RD-PNB, 29 μ M) substituents. The preference for ortho-substitution is further exemplified by L-158. N-substituents that involved para-substitutions were generally not preferred as the K_M for L-151 (91 μ M) is over 3-fold larger than that for L-150 (25 μ M). The affinity of substrates towards the enzyme was inversely related to the turnover rate as decreasing K_M values were associated with decreases in the V_{max} or k_{cat} values for each substrate (Table 1). Although L-152 was not hydrolyzed, it inhibited the hydrolysis of RD-PNB substrate with a K_i of 190 μ M.

Docking analysis reveals highly complementary enzyme-polyisoprenylated cysteinyl ester substrate interactions

While no crystal structure exists for porcine liver PMPMEase, human carboxylesterase 1 has been co-crystallized with ethyl acetate in the active site [33]. Although the K_M data obtained with the porcine isoform had been the main parameter for gauging the relative affinity of the substrates, docking analysis with Arguslab facilitated the determination of the relative binding energies for the N-, S- and O-substituted analogs to the human enzyme. As shown in Table 2, the relative binding energies follow the same general profiles as the Michaelis-Menten constants for the variations of the S-substitutions. When the N- and O-substituents were the same and the S-substituents varied, significant and systematic differences in AScore docking energies and interactions with active site residues were observed. As shown in Table 2, increasing the size of the S-alkyl substituent from ethyl to *all trans*-geranylgeranyl resulted in decreased AScore docking energies that correspond with increasing binding affinities. Consistent with the biochemical analysis that employed the porcine enzyme, the docking energies (AScore values) were in the order ethyl (L-74) < prenyl (L-77) < *trans*-geranyl (L-72) < *trans,trans*-farnesyl (RD-PNB) < *all trans*-geranylgeranyl (L-80) (Table. 2). As expected, the *all trans*-geranylgeranyl group is embedded within the hydrophobic amino acids in the active site (Fig. 2). This progressive increase in binding affinities can be attributed to the increased interactions between the increasingly larger S-alkyl groups and active site hydrophobic amino acid side chains (Fig. 3 and Table 3). The hydrophobic active site residues include Ser221, His468, Glu354, Gly142 and 143, Ile 359, Leu 97, 255, 304, and 363, Phe426, and Val146 and 254. While L-80 with the largest S-alkyl group is able to “snake” around the active site to interact with these residues, RD-PNB whose S-alkyl group is 5-carbon atoms less is comparatively limited in the number of interactions (Fig. 2, Fig. 3 and Table 3). The other substrates with increasingly shorter S-substituents display progressively lower binding affinities with the decreasing S-alkyl sizes showing correspondingly fewer active site interactions (Fig. 2).

S-alkyl bond saturation and modifications affect the affinity of substrates. A 3-fold difference in K_M was observed between L-77 with an S-prenyl (5-carbon unsaturated substituent) and L-81 which has an S-2-methyl butane substituent. This difference in affinity is shown in the AScore docking energies for L-77 and L-81 of -13.48 and -12.61 kcal/mole, respectively. Small differences in the AScore docking energies between L-72 and the two partially saturated enantiomers (L-83 and L-86) mirror the observations in their respective K_M values. The AScore binding energies for the O-alkyl substitutions also reflect the K_M values for these substrates as the AScore binding energies decreased with increasing O-alkyl size from RD-PNB (-16.15 kcal/mole) through L-76 (-16.48 kcal/mole) to L-75 (-18.65 kcal/mole). The effect of N-substitution is more ambiguous as L-152 was not metabolized and the AScore binding energies correlate less with the K_M values. Of the N-nitrobenzoyl substituents, the 2-nitro (L-157, -17.59 kcal/mole) is preferred over 3-nitro (L-156, -15.90 kcal/mole) and 4-nitro (RD-PNB, -16.15 Kcal/mole) groups. L-155 with the 3,5-dinitrobenzoyl substituent displayed a relatively low value that was not reflected in the corresponding AScore binding energy. It thus appears that the Michaelis-Menten constants obtained with porcine liver PMPMEase correlate well with the docking energies only for the S- and O-substituted substrates. When the AScore binding energies for 1YAH, PLE, 1K4Y and 1MX5 structures were plotted against the pK_M for the S-alkylated substrates, r^2 values of 0.7499, 0.7003, 0.7407 and 0.8266 were observed for the respective structures. These are higher than those for the N-substituted compounds in which r^2 values of 0.05273, 0.02796, 0.4108 and 0.1571 were observed (Fig. 4). The 1K4Y crystal structure is that obtained with a bound product from the hydrolysis of the anticancer drug CPT-11 [37]. This indicates that the K_M values obtained with porcine liver PMPMase correlate well with the AScore binding

energies obtained with the human enzyme only as far as the S-substituted analogs are concerned.

Discussion

PP enzymes have served as anticancer drug targets for over a decade. However, much of the effort has been to develop potent farnesylation inhibitors. While PPMase has received some attention [40], PMPMEase has not been evaluated as a potential target for anticancer drug development. This may be due largely to the fact that while a polyisoprenylation-dependent esterase has long been predicted, it has only recently been purified and identified [27]. PMPMEase purified from porcine liver is a serine hydrolase which is highly homologous to hCE1 [27]. The crystal structures of hCE1 reveal a large, flexible and hydrophobic active site [30]. We hypothesized that these features may be adaptations for interacting with the hydrophobic polyisoprenyl cysteinyl methyl ester at the carboxyl ends of polypeptides of various shapes and sizes [28]. Recently, a series of substrates were used to demonstrate its requirement of polyisoprenylation for high affinity interactions [28]. This is further demonstrated in the current study whereby substrates with the *S-trans*, *trans*-farnesyl group consistently show high affinity interactions with the enzyme. Furthermore, the geranylgeranylated and farnesylated substrates have the lowest binding energies as the polyisoprenyl groups interact with the hydrophobic amino acids in the active site. In addition to the AScore binding energies, the orientation of the polyisoprenyl moiety within the active site and position of the ester bond is a strong indication that PPs have co-evolved with hCE1/PMPMEase so that during catalysis, the polyisoprene is embedded in the hydrophobic binding site while the ester group on the terminal cysteine interacts with the catalytic triad near the exit. With this arrangement, the polypeptide extends away from the terminal polyisoprenylated methyl esterified cysteine and out of the active site as depicted by the position of the N-4-nitrobenzoyl group just outside of the active site. The N-4-nitrobenzoyl group substitutes the polypeptides in the substrates.

The dichotomy in the correlation between the K_M data obtained with porcine liver PMPMEase and the AScore docking energies obtained using the crystal structure of the human isoform for the S- compared to the N-substituted substrates may reflect the co-evolutionary histories of the respective enzymes and their species-specific endogenous substrates. Since the *S-trans*, *trans*-farnesyl and *S-all trans*-geranylgeranyl moieties are the standard S-substituents in the estimated 2% of eukaryotic proteins that are polyisoprenylated, the enzyme active site residues that interact with these moieties may be well conserved between species. The conservation may thus be reflected in the higher r^2 values when the pK_M values are plotted against the docking energies. The lower r^2 values for the N-substituents may be expected given the inter- and intra-species variability of the polypeptide portions of PPs that bear the polyisoprenes. As earlier stated, the N-substituents occupy the analogous positions of the polypeptides on the PPs substrates. The enzymes might have been more prone to evolutionary tolerance at sites that interact with the variable polypeptide portions than with the conserved polyisoprenyl tails. The difference between the AScore docking energies obtained with the 1K4Y crystal structure compared to the 1YAH, PLE and 1MX5 probably reflects the fact that the 1K4Y structure is that of a conformation with bound product rather than an ester substrate [37]. It may also be a reflection of conformational constraints imposed by this particular ligand. These suggest that the polyisoprenyl group may play a pivotal role in the development of high affinity and selective inhibitors of these enzymes.

Defective PP enzymes and PPs function is associated with diseases on both ends of the cell viability spectrum. For example, macular degeneration in choroideremia [11] is linked to a deficiency in geranylgeranylation caused by defective Rab-escort protein 1 (REB-1)

[11,41,42]. Knockout of the REB-1 gene results in extensive degeneration of retinal pigment epithelium and lethality [12]. PPMTase knockout in mice is lethal by mid-gestation [6,38] while the endoprotease knockout results in cardiomyopathy [43]. The polyisoprenylation pathway proteins are therefore believed to play critical developmental roles [12]. On the opposite end of these degenerative effects are the observations that excessive activity of some monomeric G-proteins is associated with various cancers [10]. The mutations of Ras and overexpression of Rab and Rho G-proteins compromise their ability to function as molecular switches [44]. In order to control their activities, farnesyl transferase has been targeted for anticancer drug development [21]. The initial promise for these compounds has not been borne out through clinical trials [24,25] as geranylgeranylation substitutes for farnesylation when farnesyl transferase inhibitors are administered [21]. Other enzymes of the pathway may therefore offer more useful targets for the development of clinically more effective compounds. Since the last reaction is the only reversible step of the pathway and believed to be of regulatory significance, it may offer better targets for the effective control of PPs function. While PMPMEase knockouts have not been reported, depletion of its activity would alter the equilibrium towards a build-up of methylated PPs.

These studies have significant implications for anticancer drug development, given the impact of hyperactive monomeric G-proteins in the etiology of several cancers. The compatibility of the active site with the polyisoprenylated substrates strongly suggests that incorporation of the polyisoprenyl moieties in inhibitors design will impart selectivity and increased affinity. The selective nature of the polyisoprenyl moiety is reflected in previous studies from this laboratory in which polyisoprenylated substrates were not hydrolyzed by acetyl- or butyrylcholinesterases [28]. The results also indicate that hCE1 serves a pertinent endogenous function in addition to its ability to metabolize ester drugs and xenobiotics [29,30].

Conclusion

The results demonstrate that the polyisoprenyl tail is the most compatible element with active site residues and is therefore the most important determinant for the selective interaction of polyisoprenylated substrates with PMPMEase. The data reveal the pertinent active site residues required for the interactions, paving the way for the design of compounds with the complementary functional groups to serve as high affinity and selective PMPMEase inhibitors. Given that PMPMEase is a key, understudied PP enzyme, such inhibitors may have wide applicability in the treatment of diseases involving polyisoprenylation pathway defects such as cancers.

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List of Abbreviations

DCC	dicyclohexyl-carbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
DMSO	dimethylsulfoxide
FCM	<i>trans, trans</i> -farnesyl-L-cysteine methyl ester
hCE1	human carboxylesterase 1 (human isoform of PMPMEase)
K_M	Michaelis-Menten constant
PLE	porcine liver esterase (porcine liver PMPMEase)
PMPMEase	polyisoprenylated methylated protein methyl esterase
PP	polyisoprenylation pathway
PPMTase	polyisoprenylated protein methyl transferase
PPs	Polyisoprenylated proteins
RD-PNB	L-N-(4-nitrobenzoyl)-S- <i>trans,trans</i> -farnesyl-cysteine methyl ester
REB-1	Rab-escort protein 1
RP-HPLC	reversed phase-high performance liquid chromatography
RT	room temperature
TEA	triethylamine
UV	ultraviolet
V_{max}	maximal velocity

Fig. 1. Michaelis-Menten and double reciprocal plots for the hydrolysis of substrates by PMPMEase

PMPMEase (0.1 to 5 μg of protein) was incubated with varying concentrations of substrates. The reactions were stopped with methanol, processed and analyzed by reversed-phase HPLC as described in the methods section. The graphs show the Michaelis–Menten kinetics (panel A) and double reciprocal (panel B) analyses for the hydrolysis of substrates based on the amounts of products formed. The results are the means ± SEM of triplicate determinations.

Fig. 2. Overlapped representation of hCE1 and modeled porcine liver PMPMEase

The crystal structure of hCE1 (1YAH) was used as template to develop a structural model for the porcine liver enzyme (PLE) using the MODELLER program as described in the methods section. Panel A shows the overlapping cartoon ribbon structure of hCE1 determined by X-ray crystallography (1YAH [33], red) and the modeled porcine homolog (yellow). Panels B-D show the Licorice models of L-80 in the active site of hCE1 (1YAH) in pink cartoon ribbon. Catalytic amino acids (Ser 221, Glu 354, His 468) are shown in blue (surf/licorice representation) while the binding site residues are in depicted in green (surf). The 4-nitro group of L-80 which is depicted by the probe lies at the entrance to the active site.

Fig. 3. Active site of hCE1 showing the interactions between substrates with varying S-alkyl substituents and the amino acids lining the binding site

The panels showing the substrates (in cylinder) at the active site are (A) L-77 with S-prenyl, (B) L-72 with S-trans-geranyl, (C) RD-PNB with S-trans,trans-farnesyl and (D) L-80 with S-all trans-geranylgeranyl substituents. The catalytic triad of amino acids (Ser221, Glu354 and His468) are shown in dark blue (surf/licorice representation) and the active site residues are in light blue licorice.

Fig. 4. Correlation between Michaelis-Menten constants and AScore docking energies for the S- and N-substituted analogs

The AScore docking energies obtained with hCE1 (1YAH and 1MX5), porcine liver esterase (PLE) and Rabbit esterase (1K4Y) were plotted against the pK_M values (-log of the Michaelis-Menten constants in molar units) obtained with porcine liver esterase for the S-substituted (A) and N-substituted (B) substrates.

Scheme 1. Synthesis of N-substituted S-farnesyl-L-cysteine methyl ester substrates

Table 1
 Michaelis-Menten kinetics analysis of N-substituted S-*trans*, *trans*-farnesylated cysteine methyl ester analogs

Compound	Structure	K_M (μ M)	V_{max} (nmol/s/mg)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)
L-150 (1)		25 ± 4.3	5.84 ± 0.295	0.362 ± 0.019	3980
L-151 (2)		91 ± 12	126.6 ± 3.02	7.85 ± 0.19	86000
L-152 (3)		Not metabolized/Inhibits RD-PNB metabolism			
RD-PNB (4)		29 ± 2.2	1.06 ± 0.03	0.07 ± 0.002	2400
L-156 (5)		35.5 ± 6.7	2.232 ± 0.076	0.1383 ± 0.0047	3897
L-157 (6)		14.0 ± 1.6	3.443 ± 0.084	0.2135 ± 0.0052	15200
L-155 (7)		5.3 ± 0.84	0.13 ± 0.0025	0.0081 ± 0.0002	1520
L-158 (8)		12.65 ± 1.54	3.10 ± 0.085	0.1922 ± 0.01	8700

* k_{cat} assumes a molecular weight for PMPMase (EPL) of 62,000 that corresponds to the translated cDNA sequence.

Table 2

Michaelis-Menten constants* and Ascore docking energies of *trans*, *trans* N-substituted S-alkylated cysteine methyl ester analogs

Compound	Structure	K_M (μ M)	AScore Docking energy (kcal/mole)			
			IYAH	PLE	1K4Y	1MX5
L-102		400 $\pm$ 115	-9.45	-8.48	-7.67	-8.36
L-111		300 $\pm$ 38	-9.84	-8.60	-7.92	-8.933
L-74		505 $\pm$ 63	-11.32	-10.11	-8.87	-10.46
L-77		294 $\pm$ 25	-13.48	-11.70	-9.69	-13.00
L-72		87 $\pm$ 12	-14.88	-13.61	-10.40	-13.86
RD-PNB (L-153)		29 $\pm$ 2.2	-16.15	-14.88	-11.05	-15.68
L-80		15 $\pm$ 2.7	-16.81	-16.28	-11.71	-17.71
L-70		11 $\pm$ 0.9	-18.71	-14.55	-11.95	-15.90
L-69		21 $\pm$ 4.2	-15.26	-13.94	-10.17	-14.34
L-81		960 $\pm$ 180	-12.61	-11.71	-9.42	-11.37

Compound	Structure	K_{M1} (μ M)	AScore Docking energy (kcal/mole)			
			IYAH	PLE	1K4Y	1MX5
L-93		40.5 $\pm$ 3.7	-14.80	-13.35	-9.87	-13.92
L-83		56 $\pm$ 9.6	-14.90	-13.13	-10.78	-14.11
L-86		53 $\pm$ 7.0	-15.34	-13.31	-10.74	-13.27
L-75		5.9 $\pm$ 0.7	-18.65	-16.63	-11.23	-16.29
L-76		11 $\pm$ 0.9	-16.48	-16.75	-11.02	-15.52
RD-18 (9)		8.4 $\pm$ 0.89	-17.51	-15.07	-11.05	-15.68
BzGFCM		3.0 $\pm$ 0.4	-18.46	-18.16	-16.79	-17.88
HCFCM		3.2 $\pm$ 0.5	-19.12	-16.87	-12.39	-18.20
L-150		25 $\pm$ 4.3	-20.44	-17.53	-10.95	-19.11
L-151		91 $\pm$ 12	-20.10	-18.33	-10.54	-19.07
L-152		Not metabolized/inhibits	-19.87	-16.87	-10.83	N/A
L-156		35.5 $\pm$ 6.7	-15.90	-15.14	-7.68	-15.83
L-157		14.0 $\pm$ 1.6	-17.59	-16.31	-11.01	-16.18

Compound	Structure	K_{M1} (μ M)	AScore Docking energy (kcal/mole)			
			IYAH	PLE	IK4Y	IMX5
L-155		5.3 ± 0.84	-14.43	-14.11	-10.80	-15.02
L-158		12.65 ± 1.542	-16.21 *	-16.56	-16.80	-17.88

* Obtained from Table 1 and [28].

Table 3
Substrate-Enzyme interactions as observed by docking analysis with ArgusLab

Residue on PMPMEase	Atoms on substrate interacting with residue on PMPMEase*										
	L-80	RD-PNB	L-72	L-77	L-74	L-70	L-75	L-76	L150		
L100	O1	O1	O1	O1	O1	O1					
F101	C2	C2	C2	C2	C2	C2	C2	C2			
L97	C2	C2	C2	C2	C2	C2					
I359	H3, H4	H3, H4	H3, H4	H3, H4				H19			
M425									H4		
H468							<i>i</i> -Propyl methyl				
L304	S6, H9, H15	S6, H9, H15				H9		S6, H7	H14		
L363	H7, H8	H7, H8					H7, H8	Ethyl ester			
S305	H11										
F303	H15										
P317	H16						H13				
L318	H16					H9			H10, H20		
L388	H19, H21					H11		H12	H7, H8		
G143	H20	H20				H14	H16		H9		
L255	H19, H20	H19, H20				H19	H20	H15			
M364	H24		H14	H9		H15, H19	H17	H16	H7		
F426	H25		H15	H10				H20, H19			

* O = oxygen, H = hydrogen on the atom designated by number, S = sulfur, C = carbon. The numbered atoms for each substrate represent the corresponding atoms on L-80 as shown in the table.