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The de-guanidinylated derivative of peramivir remains a potent inhibitor of influenza neuraminidase

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ABSTRACT

The guanidine function in the potent neuraminidase inhibitor peramivir was included early on in the drug design process, and examination of X-ray structural data for the enzyme–inhibitor complex would seem to indicate that the guanidine plays a critical role in promoting binding. However, this functional group may also contribute to the poor oral availability of the drug. Given that the relative stereochemistry on the guanidine-bearing carbon in peramivir is opposite to that in zanamivir (a related neuraminidase inhibitor, for which the guanidine function is known to contribute substantially to the potency), we sought to determine the importance of the guanidine group to peramivir's overall potency. Here we report that the de-guanidinylated analogue of peramivir is only ca. 1-order of magnitude less potent than peramivir itself in two in vitro inhibition assays. This suggests that next-generation inhibitors designed to improve on peramivir's properties might profitably dispense with the guanidine function.

The influenza virus infects approximately 600,000,000 people globally each year, resulting in 250,000–500,000 'excess deaths'.[1] Notwithstanding the implementation of a World Health Organization-monitored vaccine program, influenza remains a significant global health issue. Influenza mutates rapidly, leading to the continual emergence of new strains. Because of the long lead-time required to produce the trivalent influenza vaccine (TIV) each year, vaccinated individuals remain susceptible to these new strains.[2] Treatment of influenza infections has historically relied on two classes of molecules. The first class includes the adamantanes rimantadine and amantadine, and works by blocking the M2 proton channel.[3] Unfortunately, resistance to the adamantanes is now widespread.[4]

The second, more successful, class of drugs targets viral neuraminidase (also called sialidase). Zanamivir (see Fig. 1 for structures) was developed as a structural mimic of the boat-shaped sialic acid-hydrolysis transition structure, and is effective in limiting viral replication.[5] However, the large number of heteroatoms in zanamivir's structure limits its oral bioavailability.[6] As a result, zanamivir must be administered by aerosol inhalation, and has therefore seen reduced clinical use.[6] Oseltamivir is a second-generation neuraminidase inhibitor with substantially improved oral bioavailability.[7] This drug dominates the influenza market, with sales of ~$1 billion per year. Oseltamivir is a prodrug, which is hydrolyzed in the liver to form the biologically active carboxylate.[8]

An attempt to access neuraminidase inhibitors with a core structure distinct from oseltamivir led to development of the substituted cyclopentane peramivir. A guanidine function (as in zanamivir) was included from the beginning of the drug design process, but no effort was made to control the absolute stereochemistry at

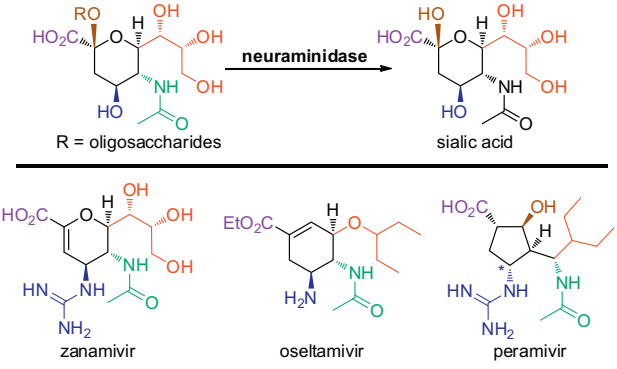

Figure 1. Function of neuraminidase, and structures of the three clinically used inhibitors. Colors indicate functionally related substituents.

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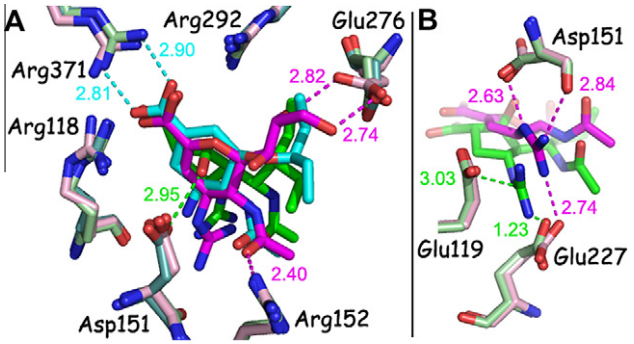

Figure 2. Comparison of binding modes and key interactions for oseltamivir (cyan; PDB 2HT8), zanamivir (magenta; PDB 2HTQ), and peramivir (green; PDB 2HTU) to a representative N8 neuraminidase (shown as lighter variants of the same color).[10] (A) top view of all three molecules in the active site. Key interactions include the carboxylate binding to the Arg118/Arg371/Arg292 triad, the peramivir hydroxyl group binding to Asp151, and the zanamivir triol binding to Glu276. A lipophilic interaction with the *N*-acetyl group is also known to be important for binding,[29] but is not shown. (B) Bottom view showing the different binding modes for the zanamivir and peramivir guanidinium groups. All distances are reported in angstroms. Images were prepared using PyMol (DeLano Scientific).

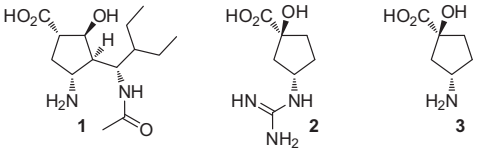

Figure 3. Structures of the three synthetic compounds evaluated in this work.

the guanidine-bearing carbon atom (indicated with an asterisk in Fig. 1).[9] The most potent compound in early development, selected from a mixture of diastereomers by co-crystallization with the enzyme, contained the opposite stereochemistry to zanamivir or oseltamivir at this centre.[9] This serendipitous discovery led to a final optimized compound (BCX-1812, which was subsequently given the name peramivir) that could more effectively probe a polar pocket at the base of the active site; as illustrated in Figure 2B for an N8 neuraminidase, the *R* guanidine function in peramivir can extend deeper into the pocket comprised of Glu119, Glu227 and the backbone carbonyl of Trp178 than can the *S* guanidine of zanamivir.[10] In other isotypes of neuraminidase, the guanidine groups in the two drugs come closer to occupying the same space in the binding site.[11] Although originally intended as an oral antiviral agent, peramivir displayed poor bioavailability in early trials, and is now being studied in formulations suitable for intravenous and intramuscular injection.[12]

Resistance of circulating influenza strains to neuraminidase inhibitors was once thought to be unlikely[13]; indeed, data prior to the 2007/2008 flu season showed resistance levels of ~1%, with somewhat higher levels in children.[14] That changed dramatically in 2007, with the report of substantial oseltamivir resistance in H1N1 strains (a common subtype of the virus) circulating in the United States (11%),[15] Canada (26%), Europe (25%) and Hong Kong (12%).[16] Even more alarming, data from the first half of the 2008/2009 flu season (prior to the emergence of the 'swine flu' H1N1 strain) showed that nearly all circulating cases of H1N1 influenza A were resistant to oseltamivir.[17] Fortunately, most cases of 'swine flu' H1N1 (e.g. A/Mexico/InDRE4487/2009) responded to treatment with oseltamivir.[18] Although not yet formally approved by the FDA, peramivir was also used successfully as an injectable antiviral agent for the treatment of the 2009 influenza A H1N1 virus.[19]

Oseltamivir resistance is largely the result of a single second-shell mutation, wherein His274 in group 1 neuraminidases (N1, N4, N5 and N8) is mutated to tyrosine. This in turn pushes a nearby carboxylate residue (Glu276) into the active site, where it suffers unfavorable interactions with the lipophilic 3-pentyl sidechain of oseltamivir, resulting in complete resistance to clinically used doses of oseltamivir. Because peramivir incorporates the same lipophilic 3-pentyl sidechain, it is likewise ineffective against the H274Y variant.[20] Strains of N1 H274Y influenza remain susceptible to zanamivir, since the triol sidechain of that drug can engage in productive hydrogen bonding interactions with Glu276 (see Fig. 2A).[21] Other mutations are known to confer resistance to zanamivir,[22] although these are currently much less common than mutations leading to oseltamivir and peramivir resistance.

The widespread prevalence of the H274Y mutation in circulating strains of influenza suggests that next-generation neuraminidase inhibitors might do well to incorporate relatively polar sidechains to bind to Glu276. This will necessarily result in an increasing number of hydrogen bond donors and acceptors, and will further decrease the log*P* to the point where such compounds are less likely to be orally active. In the hopes of regaining oral availability, it is important to understand which functional groups in the existing drugs are critically important for potency, and which groups might be replaced with less polar functionality. In this context, the carboxylic acid function common to all three drugs would seem to be required for binding to the Arg118/Arg371/Arg292 triad (see Fig. 2A), but the guanidine groups in zanamivir and peramivir represent potentially attractive sites in which to engineer improvements.

The degree to which the various functional groups on oseltamivir and zanamivir can be modified has been extensively explored in the published literature.[23–27] For example, it is known that replacement of the guanidine function in zanamivir with an amine leads to a >60-fold loss in potency (IC_{50} of 320 nM for amino-zanamivir, vs 5 nM for the drug itself)[27]; on the other hand, replacement of the amine function in oseltamivir with a guanidine leads to only a two-fold increase in potency (IC_{50} of 0.5 vs 1 nM).[23] Much less is known about the contributions to binding of the individual functional groups on peramivir. Given that the guanidine function occupies a somewhat different space for neuraminidase-bound peramivir relative to neuraminidase-bound zanamivir, and given that the guanidine binding pocket is known to accommodate a water molecule in the bound structure

Table 1
Collected data for peramivir and analogues **1–3**

Property	Peramivir	**1**
IC_{50} versus inactivated virus (10 min preincubation)	0.86 ± 0.14 nM	7.3 ± 0.7 nM
IC_{50} versus inactivated virus (2 h preincubation)	0.12 ± 0.02 nM	2.2 ± 0.9 nM
IC_{50} versus recombinant soluble neuraminidase	3.4 ± 0.4 nM	17.7 ± 2.3 nM
K_i versus recombinant soluble neuraminidase	0.83 ± 0.13 nM	7.5 ± 0.6 nM
Calculated Log*P*[38,39]	−1.37 ± 0.45	+0.33 ± 0.40
H-bond acceptors[40]	8	6
H-bond donors[40]	7	5
	2	**3**
IC_{50} versus recombinant soluble neuraminidase	4.0 ± 0.5 mM	37 ± 6 mM

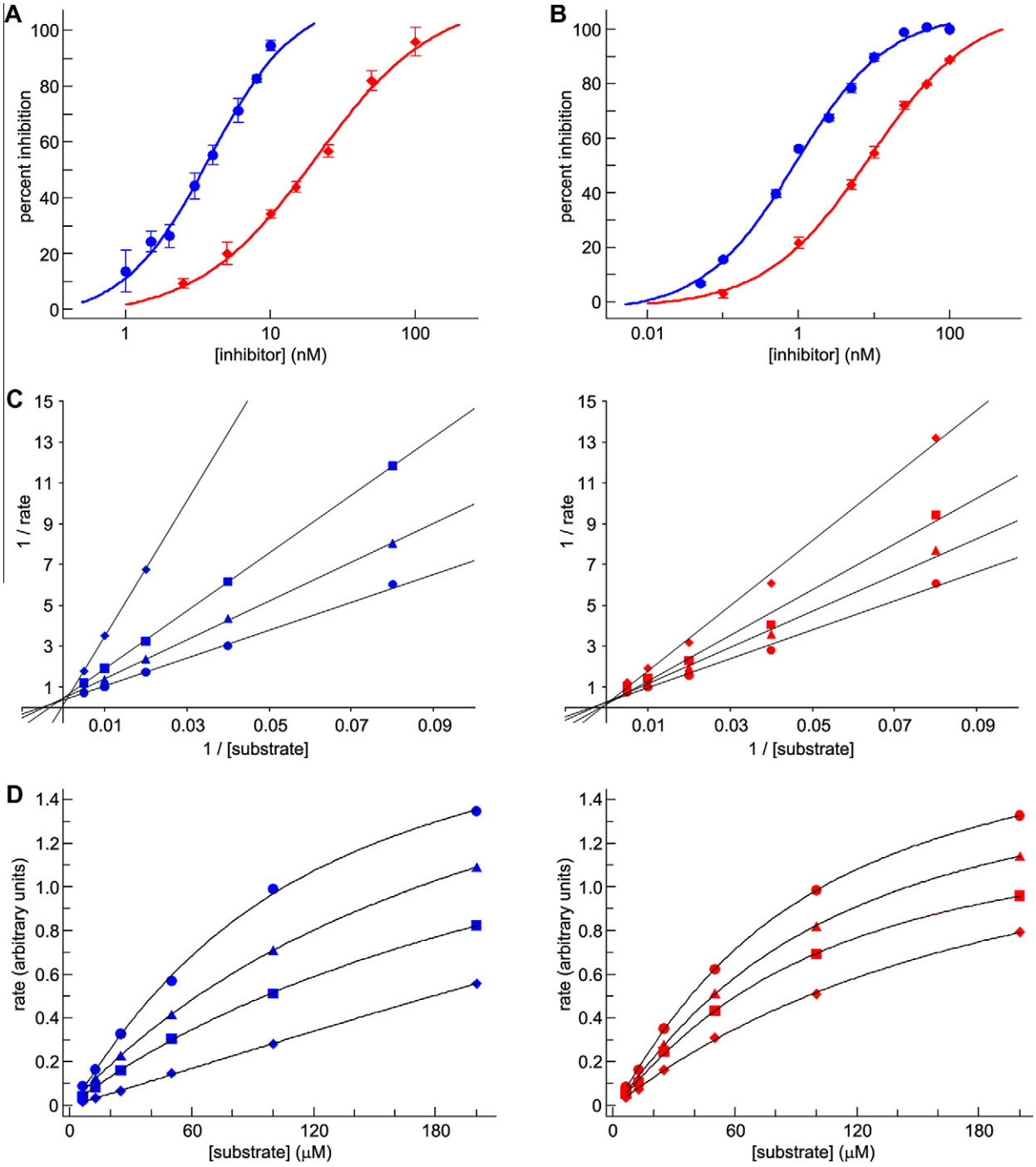

Figure 4. Inhibition data for amino-peramivir **1** (red) and peramivir (blue).[37] (A) Dose–response curve for both compounds against recombinant viral neuraminidase. (B) Dose–response curve for both compounds against neuraminidase activity in inactivated influenza virus (10 min preincubation). (C) Lineweaver–Burk representation of kinetic data at various concentrations of inhibitors; circles = 0 nM inhibitor; triangles = 0.75 nM peramivir or 3.75 nM **1**; squares = 1.5 nM peramivir or 7.5 nM **1**; diamonds = 3 nM peramivir or 15 nM **1**. (D) Michaelis–Menten representation of kinetic data at various concentrations of inhibitors. Error bars for (A) and (B) are equal to the standard error for each measurement. Error bars are not included for (C) and (D).

of oseltamivir[10] (implying that the truncation of a guanidine to an amine is well-compensated), we sought to document the activity of the amino variant of peramivir. Although this compound (**1**, Fig. 3) was reported in the original peramivir patent,[28] no detailed enzyme inhibition data was available. The extent to which the amino variant is tolerated from the standpoint of inhibitory potency may have a significant bearing on the future use of the cyclopentane scaffold to design new anti-influenza agents that better accommodate the neuraminidase H274Y mutation while maintaining or enhancing oral availability.

Amino-peramivir **1** was synthesized in six steps from commercially available (1*R*)-(−)-2-azabicyclo[2.2.1]hept-5-en-3-one[30] using a known protocol.[9b,31,32] Its ability to inhibit viral neuraminidase was evaluated relative to peramivir,[33] against a soluble recombinant neuraminidase enzyme adapted from influenza A/Brevig Mission/1/1918(H1N1),[34,35] and also against the native neuraminidase protein present in NP40-inactivated influenza A/Brisbane/59/2007(H1N1).[36]

Surprisingly, compound **1** remained a very potent neuraminidase inhibitor, with an IC_{50} of 7.3 nM against the inactivated virus

compared with 0.86 nM for peramivir, when the two test compounds were incubated with the viral solution for 10 min prior to analysis. Because zanamivir,[41] oseltamivir,[42] peramivir,[43] and other potent anti-neuraminidase agents[44] have all been reported as slow-binding inhibitors, we also compared the change in activity for peramivir and **1** upon lengthening of the incubation time to 2 h. This had the effect of increasing the apparent potency of both inhibitors. The decrease in IC_{50} for peramivir upon lengthening of the incubation period appears somewhat more substantial than the corresponding decrease for **1** (although not to a statistically significant extent), perhaps indicating that peramivir is a somewhat slower binder than is **1** (refer to Table 1 for tabulated results and estimated uncertainties at the 95% confidence interval). Measured IC_{50} values for both peramivir and **1** were slightly higher when the compounds were evaluated against the recombinant enzyme (18 nM for **1** vs 3.4 nM for peramivir), but even in this system **1** retained a K_i value in the single-digit nanomolar range (7.5 nM).

Both compounds displayed a full-range dose–response inhibitory relationship (Fig. 4A and B), and kinetic analysis revealed both molecules to be competitive inhibitors of the neuraminidase enzyme (Fig. 4C and D).[45]

A reduction in potency of a single order of magnitude (at least from such an abundantly potent inhibitor as peramivir) may be acceptable when weighed against a probable improvement in pharmacokinetic properties—particularly in cases where the remainder of the molecule is modified to include more polar functionality intended to bind Glu276. To determine whether this 1-order of magnitude loss extends to other cyclopentanes bearing an *S*-guanidine function, simplified analogues **3**[46,47] and **2**[48,47] were synthesized (also from (1*R*)-(−)-2-azabicyclo[2.2.1]hept-5-en-3-one). Once again, a roughly 10-fold loss in activity was observed in moving from the guanidine-substituted **2** to the amine-substituted **3**, despite the fact that these compounds are both far less potent than peramivir and amino-peramivir **1**.

The relatively high concentration of hydrogen bond donors and acceptors present within a single guanidine function makes these groups a potential liability in the development of orally active drugs.[49] For example, the guanidine-containing analogue of oseltamivir showed negligible oral availability in an animal model.[8] This suggests that although both the amine and guanidine functions will be charged at physiological pH, the amine is better-tolerated from the standpoint of oral availability, at least when the carboxylate function is masked as an uncharged ester. The need for next-generation inhibitors that are able to bind within the more polar active site found in H274Y neuraminidases (as for zanamivir) while maintaining oral activity (as for oseltamivir) may therefore require that compounds built on cyclopentane scaffolds (as for peramivir) forgo the inclusion of the guanidine function. The results presented here suggest that this molecular deletion is only moderately detrimental, and can likely be overcome through improvements elsewhere in the inhibitor's structure. We anticipate that this observation will be useful for the development of future neuraminidase inhibitors by our group[50] and by others in the field.[51]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.076.

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36. *Virus propagation*: Influenza A/Brisbane/59/2007 (H1N1) virus was propagated in Madin-Darby Canine Kidney (MDCK) cells. Seed virus was inoculated into 10 confluent 75 cm^2 monolayers which were incubated at 37 °C for 2–3 days and harvested when full cytopathic effect was observed. Virus purification: The pooled cell lysates were frozen and thawed and clarified by centrifugation at 3000*g* for 20 min. The supernatant was subjected to ultracentrifugation at 25,000 rpm for 90 min and the virus containing pellet was resuspended in 2 mL of MegaVir medium (Hyclone). Virus concentration was assessed by hemagglutination (HA) at 40,960 HA units. Virus inactivation: NP40 (Fluka) was added to the purified influenza virus at a final concentration of 0.2% and the mixture was incubated at room temperature for a total of 3 hours and at 4 °C for 6 h. The inactivation of the virus was confirmed by the Tissue Culture Infectious Dose assay. This virus preparation had a titre of 10^7 $TCID_{50}$ per 100 μL before inactivation and $<10^2$ $TCID_{50}$ per 100 μL after NP40 treatment.
37. The following solutions were prepared for the enzyme assays: (1) Assay buffer: 50 mM Tris, 5 mM $CaCl_2$, 200 mM NaCl, pH 7.5; (2) Protein stock solution: recombinant neuraminidase was diluted in assay buffer to 1000 ng/mL, or inactivated virus suspension was diluted to obtain a similar activity; (3) Substrate stock solution: 2′-4(methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Aldrich) was dissolved in DMSO to a concentration of 10 mM; (4) Substrate working solution: 40 μL of the substrate stock solution was diluted to 1000 μL with assay buffer, for a final concentration of 400 μM (4% DMSO); (5) Inhibitor solutions: Inhibitors were diluted in assay buffer to provide a range of working concentrations. For IC_{50} measurements with recombinant neuraminidase, sample wells of a black 96-well plate (Nunc, optical bottom) were charged with 40 μL of protein stock solution (1000 ng/mL), followed by 10 μL of inhibitor solution and 50 μL of substrate working solution (400 μM substrate, 4% DMSO). The samples (each containing 100 μL total volume, 400 ng/mL enzyme, 200 μM substrate, and 1.5% total DMSO, in 100 μL total sample volume) were mixed briefly by pipetting. Fluorescence was monitored over 5 min (λ_{exc} = 365 nm; λ_{em} = 445 nm). Experiments with inactivated virus were conducted similarly, except that the inhibitor solutions were added to wells containing inactivated virus, and these mixtures were allowed to incubate for either 10 min or 2 h at room temperature prior to the addition of working solution. For kinetic data, the working solution was subjected to serial dilution in assay buffer containing 4% DMSO. Progress of the reaction was measured over 10 min at various concentrations of substrate and inhibitor, as indicated in Figure 4. Control experiments (substrate buffer only) showed no significant background reaction. Data was plotted using XLfit (IDBS software).
38. Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.19 (© 1994-2011 ACD/Labs).
39. Log*P* values calculated by the method of Villar: −1.80 for peramivir, −0.32 for **1**; Log*P* values calculated by the method of Ghose and Crippen: −0.25 for peramivir, −0.39 for **1**.
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45. For further evidence of peramivir's ability to act as a competitive inhibitor of neuraminidase, see: Bantia, S.; Upshaw, R.; Babu, Y. S. *Antiviral Res.* **2011**, *91*, 288.
46. (1*R*,4*S*)-(−)-2-azabicyclo[2.2.1]hept-5-en-3-one (70 mg, 0.64 mmol) was heated to reflux for 18 h in methanolic hydrochloride (10 mL). The solvent was removed in vacuo to yield methyl (1*S*,4*R*)-4-aminocyclopent-2-enecarboxylate hydrochloride as a white solid. To a mixture of the crude hydrochloride salt and sodium carbonate (207 mg, 1.95 mmol) in 10 mL distilled water at 0 °C was added benzyl chloroformate dropwise via syringe (100 μL, 0.70 mmol). The solution was stirred for 30 min at 0 °C and 30 min at room temperature. The aqueous layer was extracted with 3×20 mL dichloromethane and the organic layers were combined and dried over anhydrous sodium sulfate. The solvent was removed in vacuo to yield a colorless oil. Flash-column chromatography (silica, hexanes–ether 2:1, R_f 0.22) afforded *O*-methyl, *N*-carboxybenzoyl (1*S*,4*R*)-4-aminocyclopent-2-enecarboxylate as a white solid (105 mg, 0.382 mmol, 60%). MP 155 °C; IR (cm^{-1}, film) 3428, 1731, 1716, 1506; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27–7.36 (m, 5H), 5.84–5.90 (m, 2H), 5.16 (d, J = 9.7 Hz, 1H), 5.08 (s, 2H), 4.80–4.87 (m, 1H), 3.68 (s, 3H), 3.43–3.49 (m, 1H), 2.48 (dt, J = 14.2, 8.4 Hz, 1H), 1.88 (dt, J = 14.2, 3.8 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.4, 155.8, 136.8, 134.7, 131.8, 128.7, 128.3, 128.3, 66.8, 56.5, 52.4, 49.4, 34.7. A solution of the alkene (151 mg, 0.547 mmol) and selenium dioxide (74 mg, 0.67 mmol) were heated in 15 mL anhydrous dichloromethane to 100 °C in a microwave reactor for 8 hours. The solvent was removed in vacuo to yield a brown oil. Flash-column chromatography (silica, hexanes–ether 1:2, R_f 0.10) afforded *O*-methyl, *N*-carboxybenzoyl derivative of **3** as a colourless oil (99 mg, 0.34 mmol, 62%). IR (cm^{-1}, film) 3363, 1721; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26–7.39 (m, 5H), 6.04 (d, J = 5.4 Hz, 1H), 5.74 (d, J = 5.4 Hz, 1H), 5.05–5.10 (m, 4H), 3.80 (s, 3H), 3.30 (br s, 1H), 2.43 (dd, J = 14.3, 7.6 Hz, 1H), 2.15 (dd, J = 14.3, 2.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.8, 156.1, 137.3, 136.6, 135.6, 128.8, 128.4, 128.4, 84.8, 67.0, 56.4, 53.7, 44.7. To this intermediate in 15 mL of anhydrous methanol was added 10% palladium on carbon. The mixture was stirred vigorously under an atmosphere of hydrogen gas (300 psi) for 21 h. The catalyst was removed by filtration through cotton and the filtrate was concentrated in vacuo to afford the methyl ester of **3** as a white solid (43 mg, 0.27 mmol, 97%). Mp 111 °C; IR (cm^{-1}, film) 3365, 1719; ^{1}H NMR (500 MHz, D_2O) δ 3.90–3.97 (m, 1H), 3.80 (s, 3H), 2.31–2.41 (m, 3H), 2.26 (dd, J = 14.1, 7.8 Hz, 1H), 1.86–2.00 (m, 2H); ^{13}C NMR (125 MHz, D_2O) δ 176.7, 81.2, 53.4, 50.8, 42.8, 36.8, 28.9.
47. To prepare samples for biological assays, the corresponding methyl ester was dissolved in 5% aqueous sodium hydroxide. After stirring for 4 h at room temperature, the solution was neutralized with hydrochloric acid and used directly for enzyme inhibition experiments.
48. To the methyl ester of compound **3** (22 mg, 0.14 mmol) and triethylamine (85 μL, 0.61 mmol) in 2 mL of anhydrous dimethylformamide was added 1,3-bis(benzyloxycarbonyl)-2-methyl-2-thiopseudourea (54 mg, 0.15 mmol) and mercury (II) chloride (41 mg, 0.15 mmol). After stirring for 18 h at room temperature, ethyl acetate was added and the solution was filtered through cotton. The solvent was removed in vacuo to yield a brown oil. Flash-column chromatography (silica, hexanes–ether 1:2, R_f 0.19) afforded the *O*-methyl, *N*,*N*′-bis(carboxybenzoyl) derivative of **2** as a colourless oil (30 mg, 0.064 mmol, 46%). IR (cm^{-1}, neat) 3325, 3064, 1730, 1691, 1638, 1617; ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.8 (s, 1H), 8.56 (d, J = 8.04 Hz, 1H), 7.25–7.39 (m, 10H), 5.16 (s, 2H), 5.11 (s, 2H), 4.74–4.82 (m, 1H), 3.82 (s, 3H), 3.18 (br s, 1H), 2.18–2.33 (m, 3H), 2.08 (dd, J = 14.5, 5.6 Hz, 1H), 1.82–1.88 (m, 1H), 1.72–1.80 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.2, 163.9, 155.5, 153.9, 137.0, 134.8, 128.1–129.0 (overlapping signals), 80.6, 68.5, 68.3, 53.3, 52.1, 45.9, 38.5, 32.5. To this intermediate (30 mg, 0.064 mmol) in 10 mL of anhydrous methanol was added 10% palladium on carbon. The mixture was stirred vigorously under an atmosphere of hydrogen gas (300 psi) for 24 h. The catalyst was removed by filtration through cotton and the filtrate was concentrated in vacuo to afford the methyl ester of compound **2** as a yellow oil (11 mg, 0.053 mmol, 83%). ^{1}H NMR (300 MHz, D_2O) δ 4.10–4.25 (m, 1H), 3.80 (s, 3H), 2.25–2.41 (m, 3H), 2.20 (dd, J = 14.3, 7.3 Hz, 1H), 1.75–1.94 (m, 2H).
49. For a useful review on the challenges to oral availability associated with the presence of guanidine functionality, and various strategies to mitigate these liabilities, see: Sun, J.; Miller, J. M.; Beig, A.; Rozen, L.; Amidon, G. L.; Dahan, A. *Expert Opin. Drug Metab. Toxicol.* **2011**, *7*, 313.
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51. Interestingly, when the scaffold is changed from a cyclopentane to a pyrrolidine, much less polar hydrocarbon functionality is tolerated at the position corresponding to the guanidine in peramivir; see: Ref. 44.