Supporting information for

NeuNAc oxime: A slow-binding and effectively irreversible inhibitor of the sialic acid synthase NeuB

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Equilibrium tight-binding inhibition equations

When the enzyme concentration is a significant fraction of the inhibitor concentration (i.e., when it is no longer true that $[E]_0 \ll [I]_0$), it is necessary to use the Morrison’s quadratic equation for tight-binding inhibitors (1,2):

$$v^2 + N \left[ \frac{1}{\Sigma \left( \frac{N_i}{K_i} \right)} + \frac{[I]_0 - [E]_0}{D} \right] v - \frac{N^2 E_i}{D \Sigma \left( \frac{N_i}{K_i} \right)} = 0 \quad (S1)$$

It is possible to introduce cooperative binding by adding the Hill coefficient, $n$:

$$v^2 + N \left[ \frac{1}{\Sigma \left( \frac{N_i}{K_i} \right)^n} + \frac{[I]_0^n - [E]_0^n}{D} \right] v - \frac{N^2 E_i}{D \Sigma \left( \frac{N_i}{K_i} \right)^n} = 0 \quad (S1a)$$

In the absence of cooperativity, $n = 1$. Applying the rapid-equilibrium sequential ordered ternary ternary kinetic mechanism to NeuB (3,4):

$$N = \frac{k_{cat}[Mn][PEP][ManNAc]}{K_{M,Mn}K_{M,PEP}K_{M,ManNAc}}$$
\[ N_i = 1 \]

\[ D = 1 + \frac{[\text{Mn}]}{K_{M,Mn}} + \frac{[\text{Mn}][\text{PEP}]}{K_{M,Mn}K_{M,\text{PEP}}} + \frac{[\text{Mn}][\text{PEP}][\text{ManNAc}]}{K_{M,Mn}K_{M,\text{PEP}}K_{M,\text{ManNAc}}} \]

The positive solution to the quadratic equation can be used to fit the equation:

\[ v = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \quad \text{(S2)} \]

where:

\[ a = 1 \]

\[ b = N \left[ \frac{1}{\Sigma \left( \frac{N_i}{K_i} \right)^{n}} + \frac{[I]_0^n - [E]_0}{D} \right] \]

\[ = \left( \frac{k_{\text{cat}}[\text{Mn}][\text{PEP}][\text{ManNAc}]}{K_{\text{Mn}}K_{\text{PEP}}K_{\text{ManNAc}}} \right) \times \left[ K_i^n + \frac{[I]_0^n - [E]_0}{1 + \frac{[\text{Mn}]}{K_{\text{Mn}}} + \frac{[\text{Mn}][\text{PEP}]}{K_{\text{Mn}}K_{\text{PEP}}} + \frac{[\text{Mn}][\text{PEP}][\text{ManNAc}]}{K_{\text{Mn}}K_{\text{PEP}}K_{\text{ManNAc}}}} \right] \]

\[ c = \frac{N^2E_t}{D\Sigma \left( \frac{N_i}{K_i} \right)} = \frac{\frac{-1}{2}}{\left( 1 + \frac{[\text{Mn}]}{K_{\text{Mn}}} + \frac{[\text{Mn}][\text{PEP}]}{K_{\text{Mn}}K_{\text{PEP}}} + \frac{[\text{Mn}][\text{PEP}][\text{ManNAc}]}{K_{\text{Mn}}K_{\text{PEP}}K_{\text{ManNAc}}} \right)} \times \left( \frac{1}{K_i} \right)^n \]
Figure S1. (a) $^1$H and (b) $^{13}$C NMR spectra of NeuNAc oxime.
Figure S2. Initial velocity versus variable substrate concentrations for variable (a) Mn$^{2+}$, (b) PEP, and (c) ManNAc concentrations fitted to eq. S3 for a rapid equilibrium sequential ordered ter ter kinetic mechanism (3). Eq. S3 is the same as eq. 1 of reference 3. Only a subset of data, with the highest fixed substrate concentrations is shown. The inset graphs are Hanes plots ([S]/v₀ vs. [S]).

Equation S3: $v_0 = \frac{k_{cat}[Mn][PEP][ManNAc]}{1 + \frac{K_{M,Mn}}{[Mn]} + \frac{K_{M,PEP}}{[PEP]} + \frac{K_{M,ManNAc}}{[ManNAc]}}$
Figure S3. Equilibrium model of NeuNAc oxime inhibition. (a) The rate of onset of inhibition, $k_{on}$, was fitted to the second-order integrated rate equation (equation 3), with $k_{on}(\text{apparent}) = 0.038 \pm 0.008 \text{ M}^{-1} \text{s}^{-1}$ and offset(\text{apparent}) = 0.15 ± 0.07. (b) $K(\text{apparent})$ for 20 min preincubation of NeuNAc oxime with NeuB fitted to Morrison's quadratic equation (equation S2). (black line) $K(\text{apparent}) = 6.5 \pm 1.1 \text{ μM}$, with an offset of $\nu(\text{offset}) = 0.40$. (grey line) Fitted with the cooperative model. $K(\text{apparent}) = 16 \pm 7 \text{ μM}$ and a Hill coefficient, $n = 1.4 \pm 0.3$. (c) $K(\text{apparent})$ for 18 h preincubation fitted to Morrison's quadratic equation (equation S2). (black line) $K(\text{apparent}) = 0.9 \pm 0.3 \text{ μM}$, with an offset of $\nu(\text{offset}) = 0.13$. (grey line) Fitted with the cooperative model. $K(\text{apparent}) = 1.4 \pm 0.3 \text{ μM}$ and a Hill coefficient, $n = 1.4 \pm 0.1$. (insets) Same data plotted with [NeuNAc oxime] plotted on a log scale. Relative initial velocities ($\nu = \nu(\text{inhibited})/\nu(\text{control})$) are reported to account for activity loss during extended preincubations. The offset, $\nu(\text{offset})$ is equal to the residual activity at high inhibitor concentrations. The apparent value of $\nu(\text{offset})$ for t = 20 min preincubation is not accurate since binding is not complete after 20 min.
Table S1. Dynafit models of NeuNAc oxime inhibition.
E = NeuB, I = NeuNAc oxime. All concentration units are M and time units are s⁻¹. The NeuB’s kinetic parameters are from Table 1, with the association rate constants (km, kmp, kmpm) set to the approximate diffusion rate limit of 10⁹ M⁻¹ s⁻¹, and the dissociation rate constants (k-m, k-mp, k-mpm) defined give the corresponding K_M values. For example, K_M,Mn = 8.5 × 10⁻⁴ M, therefore k-m = 8.5 × 10⁵ s⁻¹, so that k-m/km = 8.5 × 10⁸ s⁻¹ / 10⁹ M⁻¹ s⁻¹ = 8.5 × 10⁻⁴ M. In this example, k₁ = 10⁶ M⁻¹ s⁻¹, but equivalent values of K_i were obtained by fixing k₁ at 10⁸ to 10⁹ M⁻¹ s⁻¹, and fitting k-1. The cooperativity model is rudimentary, as many possible models of cooperativity could be created, but the current data would not allow discrimination between them. The progress curve in Figure 4 (dashed line) was generated using the same model and kinetic constants, with [I] = 0.

---

No cooperativity

[mechanism]

\[
\begin{align*}
E + Mn & \leftrightarrow E.Mn : \text{km} \quad \text{k-m} \\
E.Mn + PEP & \leftrightarrow E.Mn.PEP : \text{kmp} \quad \text{k-mp} \\
E.Mn.PEP + ManNAc & \leftrightarrow E.Mn.PEP.ManNAc : \text{kmpm} \quad \text{k-mpm} \\
E.Mn.PEP.ManNAc & \rightarrow E + Mn + NeuNAc : \text{kcat} \\
E + I & \leftrightarrow E.I : \text{k1} \quad \text{k-1} \\
E.I & \rightarrow E*.I : \text{k2}
\end{align*}
\]

[constants]

\[
\begin{align*}
\text{km} & = 1e9 \\
\text{k-m} & = 8.5e5 \\
\text{kmp} & = 1e9 \\
\text{k-mp} & = 4.16e6 \\
\text{kmpm} & = 1e9 \\
\text{kcat} & = 2.8 \\
\text{k1} & = 1e6 \\
\text{k-1} & = 36? \\
\text{k2} & = 5.6e-5?
\end{align*}
\]

[responses]

\[
\begin{align*}
E*.I & = 1 \\
E.I & = 1
\end{align*}
\]

Cooperativity model

[mechanism]

\[
\begin{align*}
E + Mn & \leftrightarrow E.Mn : \text{km} \quad \text{k-m} \\
E.Mn + PEP & \leftrightarrow E.Mn.PEP : \text{kmp} \quad \text{k-mp} \\
E.Mn.PEP + ManNAc & \leftrightarrow E.Mn.PEP.ManNAc : \text{kmpm} \quad \text{k-mpm} \\
E.Mn.PEP.ManNAc & \rightarrow E : \text{kcat} \\
E + I & \leftrightarrow E.I : \text{k1} \quad \text{k-1} \\
E.I + I & \leftrightarrow E.I2 : \text{k1} \quad \text{k-1} \\
E.I2 & \rightarrow E*.I2 : \text{k2}
\end{align*}
\]

[constants]

\[
\begin{align*}
\text{km} & = 1e9 \\
\text{k-m} & = 8.5e5 \\
\text{kmp} & = 1e9 \\
\text{k-mp} & = 4.16e6 \\
\text{kmpm} & = 1e9
\end{align*}
\]

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\[ k_{\text{mpm}} = 3.52 \times 10^5 \]
\[ k_{\text{cat}} = 2.8 \]
\[ k_1 = 1 \times 10^6 \]
\[ k_{-1} = 28.5? \]
\[ k_2 = 8.1 \times 10^{-5}? \]

[responses]
E*.I2 = 1
E.I2 = 1
E.I = 1

**Table S2. Phosphate-binding interactions in NeuB and DAHP synthase crystal structures.**

<table>
<thead>
<tr>
<th></th>
<th>Enzyme</th>
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</thead>
<tbody>
<tr>
<td>Phosphate bridging oxygen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NeuB (^a)</td>
</tr>
<tr>
<td></td>
<td>DAHP synthase (^b)</td>
</tr>
<tr>
<td>K129 N(\zeta)</td>
<td>K186 N(\zeta)</td>
</tr>
<tr>
<td>Phosphate non-bridging oxygens</td>
<td>Neutral hydrogen bonds:</td>
</tr>
<tr>
<td></td>
<td>S132 (O(\gamma) and backbone NH)</td>
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<tr>
<td></td>
<td>S154</td>
</tr>
<tr>
<td></td>
<td>S213</td>
</tr>
<tr>
<td></td>
<td>Equivalent of ManNAc O1 / NeuNAc O4</td>
</tr>
<tr>
<td></td>
<td>Ion pair:</td>
</tr>
<tr>
<td></td>
<td>Mn(^{2+})</td>
</tr>
<tr>
<td></td>
<td>Ion pairs:</td>
</tr>
<tr>
<td></td>
<td>R234 (bidendate)</td>
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<tr>
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<td>R165 (monodentate + H(_2)O mediated)</td>
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<tr>
<td></td>
<td>Water mediated:</td>
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<td>HOH19</td>
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<td>HOH759</td>
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<td>A164 NH</td>
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<tr>
<td></td>
<td>R165 NH</td>
</tr>
</tbody>
</table>

\(^a\) PDBID: 1XUZ (5), 2WQP (6), and 6PPZ (this study).

\(^b\) PDBID: 1N8F (7).

**References**


