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Phenylethanolamine *N*-methyltransferase inhibition: re-evaluation of kinetic data

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Abstract—Inhibitors of phenylethanolamine *N*-methyltransferase [PNMT, the enzyme that catalyzes the final step in the biosynthesis of epinephrine (Epi)] may be of use in determining the role of Epi in the central nervous system. Here we demonstrate that a routinely used assay for screening PNMT inhibitors is not appropriate for those inhibitors having K_i values less than 1 μM. A revised assay has been developed that shows some inhibitors bind two orders of magnitude more tightly than previously reported.

Phenylethanolamine *N*-methyltransferase (PNMT; E.C. 2.1.1.28) catalyzes the terminal step in catecholamine biosynthesis, that is, the conversion of norepinephrine to epinephrine (adrenaline).[1,2] Although epinephrine (Epi) makes up 5–10% of the total catecholamine content of the brain, its function within the central nervous system (CNS) is not well understood.[3] CNS epinephrine has been implicated in a variety of activities including central control of blood pressure[4] and respiration,[5,6] secretion of hormones from the pituitary[7] and may even be responsible for some of the neurodegeneration found in Alzheimer's disease.[8] Delineation of the role of central Epi will require an agent that can be used to regulate epinephrine levels within the CNS. An inhibitor of PNMT may provide such an agent that may be of potential pharmaceutical benefit.

Over the years, many inhibitors of PNMT have been developed. Unfortunately, most interact with other biologically important sites, such as the α_2-adrenoceptors, which limits their utility as pharmacological tools. The majority of those inhibitors were tested against partially purified preparations of mammalian, primarily bovine, PNMT. Recently, we described the purification of human PNMT (hPNMT) expressed in *Escherichia coli*.[9] This permitted us to compare the kinetics of a series of structurally diverse substrates and inhibitors with hPNMT and the bovine adrenal enzyme.[10] These compounds were evaluated using a standard radiochemical assay[11] that has been used for substrates[12] and inhibitors.[13] Although there were some differences in substrate/inhibitor specificity, the results showed that the conclusions from early structure–activity studies with bovine adrenal PNMT broadly held true for hPNMT.[10] Recently we obtained X-ray structures of hPNMT with several different inhibitors bound in the active site.[14,15] These structures guided a mutagenic study of the PNMT active site, the results of which will be described elsewhere. However, during this study, we observed several anomalous kinetic results with the inhibition of hPNMT mutants. These results suggested that the standard assay used for PNMT inhibitors may not be appropriate in all cases, particularly for those with sub-micromolar affinity. In light of this we have carried out a detailed re-evaluation of the standard PNMT assay, as described below. The study prompted the development of a revised assay procedure, in which several inhibitors are shown to have K_i values for hPNMT 2–100-fold lower than those previously reported.[10]

PNMT assays generally follow the transfer of either a ^{14}C- or ^{3}H-labeled methyl group from *S*-adenosyl-L-methionine (AdoMet) to norepinephrine or phenylethanolamine.[2,16] The majority of inhibitors described to date are competitive with substrate. Consequently, the inhibition assay is generally carried out at a fixed

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concentration of AdoMet with the concentrations of amine substrate and inhibitor being varied as appropriate. The standard assay described in Grunewald et al.[10] maintains AdoMet at 1 mM, while phenylethanolamine (PEA) is varied between 30 and 100, and 60 and 200 μM for bovine adrenal and human PNMT, respectively. A wide range of inhibitor concentrations was used, depending on the value of K_i but the lowest concentration used was 100 nM. The bovine adrenal enzyme was only partially purified and required 200–300 μg protein/assay (in a total assay volume of 250 μL). The exact concentration was determined experimentally for each new enzyme preparation by assessing the amount of protein required to transfer 5–10% of the ^3H label to the amine substrate, ostensibly ensuring that the kinetics were measured under initial rate conditions. The human enzyme had been purified to homogeneity[9] and required only ~4 μg/assay to meet the same requirements.

Examination of these assay conditions reveals three potential problems: (i) the percentage conversion to product is based on AdoMet, which is not the limiting substrate, (ii) relatively high concentrations of PNMT are required for timely conversion to product, and (iii) not only is hPNMT potentially susceptible to substrate inhibition, but also AdoMet often contains some *S*-adenosyl-L-homocysteine (AdoHcy) as a contaminant. We have looked at each of these, in turn, with particular emphasis on the human enzyme as it has yet to be studied in any great detail.

In Table 1 we show the effect of using sufficient hPNMT (1.25 μM) to ensure that, at 200 nM PEA, ~5% of the ^3H label is transferred to the substrate. The data clearly demonstrate that, for any concentration of PEA, less than 5% of the AdoMet (initially 1 mM) is consumed in the reaction. However, at an initial PEA concentration of 30 μM, almost 50% of the PEA is converted to product. Indeed, at all concentrations of PEA, the reaction has gone to at least 25% completion. This clearly shows that the earlier assays were not being carried out under the initial rate conditions necessary to use Michaelis–Menten kinetics for the determination of K_i values.

Potentially, the biggest problem is the concentration of enzyme used in the assay. Studies with mutant proteins suggested that the extent of inhibition was dependent on protein concentration and, subsequently, we were able to demonstrate a similar effect with wild-type hPNMT for those inhibitors with K_i values below 1 μM (data not shown). The previous study on the inhibition of hPNMT[10] employed Michaelis–Menten kinetics (and the inherent steady-state approximations) to determine K_i values. It has been suggested that this approach is not valid when the K_i value of the inhibitor is less than 1000-fold greater than the total enzyme concentration.[17,18] Given that the earlier study[10] was carried out at a hPNMT concentration of ~4 μg/assay or ca. 500 nM, this would apply to many of the inhibitors. For those inhibitors, the population of free inhibitor molecules will be significantly depleted by the formation of the enzyme–inhibitor complex, that is, the inhibitors will be effectively tight-binding, and the data should be treated according to Eq. 1.[18]

$$\frac{v_i}{v_0} = 1 - \frac{([E] + [I] + K_i^{app}) - \sqrt{([E] + [I] + K_i^{app})^2 - 4[E][I]}}{2[E]} \quad (1)$$

Here, v_0 and v_i are the reaction rates in the absence and presence of inhibitor, respectively, $[E]$ is the total enzyme concentration, $[I]$ is the inhibitor concentration, and K_i^{app} is the apparent enzyme–inhibitor dissociation constant. As demonstrated in Table 2, this is not a major problem for the weaker inhibitors as errors brought about by the steady-state treatment of data do not become really significant until $K_i/[E]$ is less than five.

Conversely, for better inhibitors, not only will the K_i data obtained using a steady-state treatment of data have large errors, it may also be very misleading. For example, if an inhibitor is tight binding, it will interact with the enzyme in a nearly stoichiometric manner. Therefore, as the enzyme concentration increases, more

Table 2. Effect of ratio $K_i/[E]$ on observed inhibition

$K_i/[E]$	v_i/v_0[a]	%Error in v_i/v_0[b]
100	0.501	0.2
20	0.506	1.2
10	0.513	2.5
5	0.525	5.0
2	0.562	12.3
1	0.618	23.6
0.5	0.707	41.4
0.2	0.839	67.7
0.1	0.910	82.0

[a] Values calculated using Eq. 1 assuming no substrate is present and $[I] = K_i$. Under these conditions, if $K_i \gg [E]$, $v_i/v_0 = 0.5$.
[b] %Error $= (v_i/v_0 - 0.5)/0.5 \times 100$.

Table 1. Extent of reaction as a function of substrate concentration

[PEA] (μM)	PEA (pmol/assay)	dpm/assay[a,b]	pmol/30 min[c]	%Reaction of AdoMet	%Reaction of PEA
200	50,000	6841	12,440	4.9	24.7
150	37,500	6223	11,314	4.5	30.0
100	25,000	4961	9021	3.6	35.9
60	15,000	3561	6475	2.6	42.9
30	7500	2065	3754	1.5	49.7

[a] Assays were carried out at 1 mM AdoMet, with 277,500 dpm methyl ^3H AdoMet per assay as described in Ref. 10.
[b] Values are mean of three experiments and reflect the number of dpm in the product per 1 mL of organic layer.
[c] Data not corrected for extraction efficiency.

inhibitor will be required to attain the same level of inhibition. This is described by Eq. 2.[19,20]

$$IC_{50} = 0.5[E] + K_i^{app} \quad (2)$$

At an enzyme concentration of 500 nM, inhibitors with apparent K_i values of 1, 10, and 100 nM will have broadly similar IC_{50} values (251, 260, and 350 nM, respectively). Clearly, under these assay conditions, it is almost impossible to obtain meaningful IC_{50} values, and it suggests that the similar sub-micromolar K_i values reported earlier represent, in effect, a titration of the enzyme rather than true values of K_i.

To some extent the problems described above result from the use of a high concentration of AdoMet in the assay. The ratio of labeled/unlabeled AdoMet is very low, making the assay relatively insensitive. There are also some other undesirable consequences. It has been demonstrated that PNMT from a variety of species is subject to substrate inhibition by norepinephrine.[21,22] Inhibition by AdoMet is less common, but it has been reported for some isozymes of rabbit adrenal PNMT.[22] However, the studies examining substrate inhibition by AdoMet were carried out at concentrations of less than 100 μM, significantly below those in the assay under review.[21–23] Further, and more worrying, PNMT is strongly inhibited by its reaction product, AdoHcy with K_i values in the low micromolar range. Commercial preparations of AdoMet are well known to contain small amounts of AdoHcy.[24,25] Even if the contamination was as little as 1%, an assay using 1 mM AdoMet would contain at least 10 μM AdoHcy, which is above the K_i values reported for AdoHcy inhibition of rabbit adrenal PNMT.[24,26] We therefore examined the effect of AdoMet concentration on the reaction catalyzed by hPNMT.

The results, shown in Figure 1, clearly demonstrates that high concentrations of AdoMet do have an inhibitory effect on hPNMT catalysis. It is beyond the scope of this work to determine whether this is due to substrate or product inhibition. While this in itself may not be a fatal flaw in assay design, as reactions without inhibitor are run under identical conditions, it is still not optimal to have results clouded by inhibition from sources other than the inhibitor under investigation.

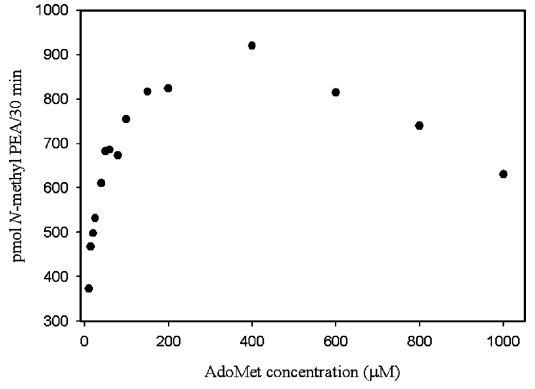


Figure 1. Effect of AdoMet concentration on the reaction catalyzed by hPNMT. The initial phenylethanolamine concentration was 200 μM and the hPNMT concentration was 63 nM.

Overall, the data suggest that the current assay is not optimal for all inhibitors, and is certainly inappropriate for the assay of sub-micromolar inhibitors. Overcoming these problems requires a reduction in the assay concentrations of both AdoMet and hPNMT. Initially, the AdoMet concentration was lowered to 5 μM ($\sim K_m$), while the concentration of labeled AdoMet was maintained at ca. 300,000 dpm/assay. This had the dual effect of increasing the sensitivity of the assay and guaranteeing that, if the amount of product formed (in dpm) is <5–10% of the initial number of counts, the assay will have been carried out under initial rate conditions. The increase in sensitivity permitted the hPNMT concentration to be reduced to 30 ng/assay ($\sim$4 nM), thereby ensuring that all but the most potent inhibitors can be routinely assayed and the data fit to Eq. 3.

$$v = \frac{V_{max}[S]}{[S] + K_m\left(1 + \frac{[I]}{K_i}\right)} \quad (3)$$

For the most potent inhibitors, even an enzyme concentration as low as 4 nM is still too high to use steady state approximations. In those cases the inhibitor must be considered to be tight binding and the inhibition data should therefore be fit to Eq. 1.

We have used the modified assay[27] to re-evaluate those inhibitors previously reported[10] as having K_i values for hPNMT of less than 1 μM. The results are shown in Table 3.

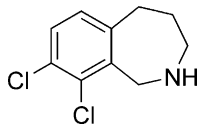
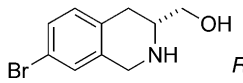
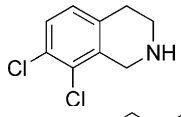
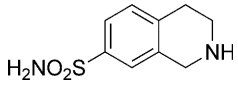
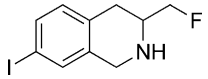
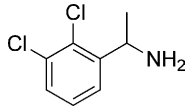
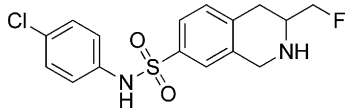
The results show that, in all cases, a lower value of K_i was obtained using the revised assay. The increase in binding affinity ranged from ca. 2- to 100-fold and, not unexpectedly, the greatest increase was shown by those compounds with the lowest values of K_i in the original assay. Whilst these results do not change the structure–activity conclusions reached in the earlier paper,[10] we are now able to discern considerable differences between inhibitors whose K_i values were essentially identical in the previous study.

We have now adopted a 2-fold approach to determining K_i values for new hPNMT inhibitors. First an IC_{50} value for the inhibitor is obtained at a phenylethanolamine concentration of 100 μM (i.e., at K_m), with AdoMet and hPNMT concentrations of 5 μM and 4 nM, respectively. For a competitive inhibitor, the value of K_i is related to IC_{50} by Eq. 4.[28] For those compounds with an IC_{50} value above 100 nM, the K_i value is then determined by fitting to Eq. 3 using variable PEA and inhibitor data obtained at AdoMet (5 μM) and hPNMT (4 nM).

$$IC_{50} = K_i^{app}\left(1 + \left[\frac{[S]}{K_m}\right]\right) \quad (4)$$

For inhibitors with IC_{50} values below 100 nM, $K_i^{app}/[E]$ will be <12.5. The inhibitor is therefore considered to be

Table 3. Comparison of K_i values obtained using original and revised assay[a]

Compound[b]	K_i hPNMT[c]	K_i This work[d]
	0.28 (0.03)	0.012 (0.001)
	0.30 (0.03)	0.0049 (0.0006)
	0.30 (0.04)	0.0031 (0.0006)
	0.58 (0.04)	0.28 (0.02)
	0.66 (0.07)	0.073 (0.004)
	0.81 (0.09)	0.090 (0.006)
	0.95 (0.09)	0.27 (0.03)

[a] Values of K_i are expressed in μM (SEM).
[b] Compound numbers from Ref. 10 are **12**, **18**, **19**, **21**, **24**, **13**, and **26**, as listed from top to bottom.
[c] Data from Ref. 10.
[d] Assayed as described in Ref. 27. K_i values were determined by a hyperbolic fit of kinetic data to Eq. 1.

tight binding, and the variable PEA and inhibitor data are fit to Eq. 1.

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27. The revised inhibition assay comprises phosphate buffer (50 mM, pH 8.0), unlabeled AdoMet (5 μM), [methyl–^{3}H]AdoMet, (ca. 3×10^5 dpm), hPNMT (~4 nM), phenylethanolamine, and inhibitor in a final volume of 250 μL. Inhibition assays were run using four–five concentrations of both phenylethanolamine and inhibitor, both over a 10-fold range, and reaction workup was as described previously.
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