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THE INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE BY 2,3-DIHYDROXYPYRIDINE

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Abstract—Despite its structural similarity to catechol, 2,3-dihydroxypyridine is not a substrate but a "dead-end" inhibitor of purified pig liver catechol-*O*-methyltransferase. It inhibits the methylation of 3,4-dihydroxyphenylacetic acid competitively with an inhibitor constant of 15 μM. Against the methyl donor, *S*-adenosyl-L-methionine, it is an uncompetitive inhibitor (K_i' = 85 μM). Clearly, although 2,3-dihydroxypyridine interacts with the catechol-binding site of the enzyme, the presence of a nitrogen in the ring alters its susceptibility to *O*-methylation.

Catechol-*O*-methyltransferase (COMT)(EC 2.1.1.6) is a widely distributed enzyme involved mainly in extraneuronal catabolism of catecholamines (see Refs. 1 and 2 for review). The possession of a specific and potent inhibitor of COMT has long been a goal of investigators striving to elucidate the physiological role and properties of the enzyme. Whilst a large number of COMT inhibitors are known (see Refs. 3 and 4 for review), most of them suffer from lack of specificity and the large number of potential side-effects has prevented their use in clinical medicine. This is unfortunate since altered methyltransferase activity has been implicated in schizophrenia [5, 6] and affective disorders (see Ref. 7 for summary) and a COMT inhibitor may be beneficial in the therapy of Parkinson's disease [8].

COMT inhibitors have, however, found employment in isolated tissue preparations (see, for example, Ref. 9). The most widely used is U-0521 (3′,4′-dihydroxy-2-methylpropriophenone) which has been shown to competitively inhibit COMT from a variety of tissues [10–12]. U-0521, however, suffers from such drawbacks as limited *in vivo* effectiveness, β-adrenoceptor aganist activity [13], and inhibition of tyrosine hydroxylase [14].

Thus, if a specific, unambiguous inhibitor of COMT were found it could yield much information about the role of COMT under normal, disordered and disease states. The inhibitor would also be of use for *in vitro* mechanistic studies.

The investigation of 2,3-dihydroxypyridine (2,3-pyridinediol) as a prototype COMT inhibitor was prompted by the findings of Borchardt [15] that structurally related pyrones and pyridones acted as "dead-end" inhibitors of COMT. 2,3-Dihydroxypyridine should have an advantage over the latter compounds as its vicinal hydroxyl groups will confer upon it a high affinity for the active site of COMT. The nitrogen in the pyridine ring might alter it susceptibility to methylation.

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MATERIALS AND METHODS

Unless otherwise stated, all reagents were of analytical quality (BDH, Poole, U.K.) and were dissolved in glass-distilled water. 2,3-Dihydroxypyridine was obtained from Aldrich (Gillingham, U.K.). All pH measurements were carried out at 20°.

COMT was purified from deep-frozen pig liver by homogenisation, ammonium sulphate precipitation from the supernatant, chromatography of the redissolved precipitate on Sephadex G75 and affinity chromatography on 2,6-dimethoxyphenol-azophenyl-methylene-anilino-agarose as in Ref. 16 but with modified buffers [17]. During purification COMT activity was assayed by the direct-extraction radiochemical method of Zürcher and Da Prada [18] using catechol as the methyl acceptor and methyl-tritiated *S*-adenosyl-L-methionine (AdoMet) (Amersham, U.K.) as the methyl donor in 0.08 M potassium phosphate buffer (pH 7.6). One unit (U) of activity represents the formation of 1 μmole of product in 1 min at 37°.

"Non-specific" adenosine deaminase (EC 3.5.4.4) was partially purified from Takadiastase (Koch-Light, Colnbrook, U.K.) using the method of Sharpless and Wolfenden [19] modified to use 150 g Takadiastase but replacing the dialysis stage with a 500-cm^3 bed-volume column of Sephadex G25 equilibrated and running in 0.01 M potassium acetate buffer (pH 5.3).

The coupled spectrophotometric assay of Coward and Wu [20] was used to study the inhibition of COMT by 2,3-dihydroxypyridine. The assay was modified as in Ref. 21 so that the mixture contained 1.6 mM magnesium chloride, 0.64 I.U. adenosine deaminase, 0.2 M triethanolamine hydrochloride buffer (pH 7.2), AdoMet, 3,4-dihydroxyphenylacetic acid (DOPAC), 2,3-dihydoxypyridine dissolved in up to 20 μl methanol and 10 μl (22.2 mU) purified COMT in a final volume of 500 μl. The volume of methanol alone had no effect on the catalytic rate observed.

In experiments to investigate the kinetics of inhibition against DOPAC, the concentration of

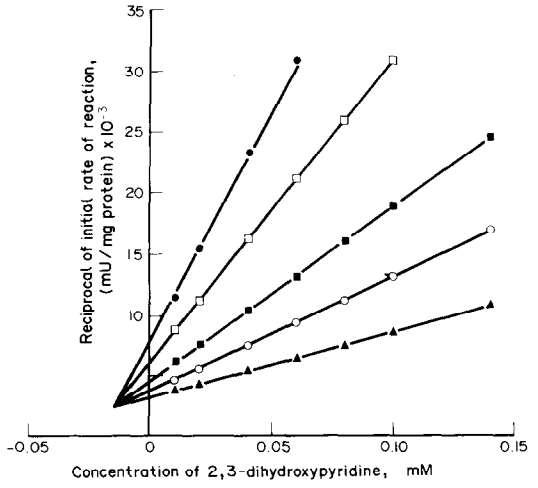

Fig. 1. Assayed by the method of Coward and Wu [20] as modified in Ref. 21. Assay carried out in the presence of 1.6 mM $MgCl_2$, 0.456 mM AdoMet and 0.2 M triethanolamine hydrochloride buffer (pH 7.2) at 37°. Key: Concentration of 3,4-dihydroxyphenylacetic acid (mM): ●, 0.15; □, 0.30; ■, 0.45; ○, 0.60; ▲, 0.96.

AdoMet was fixed at 0.456 mM. Similarly, in experiments in which AdoMet was the varied substrate, the cuvette concentration of DOPAC was maintained at 3 mM. The concentration ranges used were: 2,3-dihydroxypyridine, 0.01–0.386 mM; AdoMet, 0.04–0.456 mM; and DOPAC 0.15–0.96 mM.

Initial rates of decrease in absorbance at 265 nm were measured either in the presence or absence of 2,3-dihydroxypyridine at 37° in a Beckman model 35 spectrophotometer. Each point on the inhibition plots (see Figs. 1–3) represents the median kinetic rate of at least eight replicates.

RESULTS AND DISCUSSION

Results of the purification of COMT are given in Table 1. The sp. act. of COMT in the initial homogenate is high, accounting for the apparently low absolute degree of purification. However, the sp. act. of the pooled affinity chromatography fractions used in the inhibition studies is also higher than that obtained previously by this technique [16, 17, 22]. Polyacrylamide gel electrophoresis detected one protein species only.

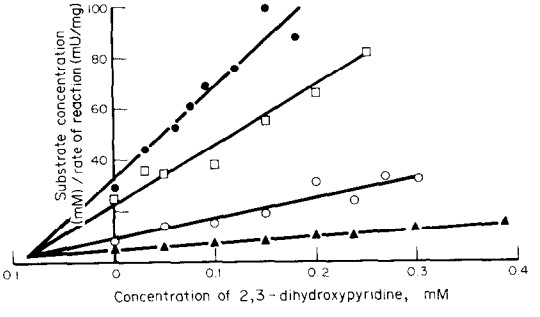

Fig. 2. Assayed by the method of Coward and Wu [20] as modified in Ref. 21. Assay carried out in the presence of 1.6 mM $MgCl_2$, 3 mM 3,4-dihydroxyphenylacetic acid and 0.2 M triethanolamine hydrochloride buffer (pH 7.2). The plot was drawn using calculated lines of best fit. Key: Concentration of AdoMet (mM): ●, 0.456; □, 0.276; ○, 0.108; △, 0.040.

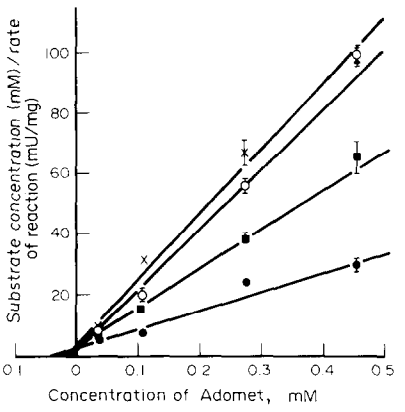

Fig. 3. Assayed by the method of Coward and Wu [20] as modified in Ref. 21. Assay carried out in the presence of 1.6 mM $MgCl_2$, 3 mM 3,4-dihydroxyphenylacetic acid and 0.2 M triethanolamine hydrochloride buffer (pH 7.2). Key: Concentration of 2,3-dihydroxypyridine (mM): ●, 0.00; ■, 0.10; ○, 0.15; ×, 0.20.

2,3-Dihydroxypyridine was not found to be a substrate for COMT. Assays in which 2,3-dihydroxypyridine was utilised as a putative substrate, replacing DOPAC, did not result in any *O*-methylation of 2,3-dihydroxypyridine with a cuvette concentration of up to 2 mM.

Inhibition of DOPAC O-methylation

2,3-Dihydroxypyridine was, however, found to be an inhibitor of COMT: the results obtained for studies in which the concentration of DOPAC was varied in the presence of saturating AdoMet are expressed as a Dixon plot [23] (Fig. 1). This shows 2,3-dihydroxypyridine to be a competitive inhibitor of DOPAC methylation and to have an inhibitor constant (K_i) of 15 μM (S.E.M. ± 0.02 μM. This latter value was calculated from the variation within replicate observations.) The K_i value is of the same order as that of pyrogallol (K_i = 8 μM against noradrenaline [24]) and quercetin (K_i = 5.3 μM against noradrenaline [25]) and compares favourably to that for pyridoxal-5'-phosphate (K_i = 53.9 μM against noradrenaline [26]) and multi-substrate adducts (K_i in millimolar range against 3,4-dihydroxybenzoic acid [27]). It would seem that 2,3-dihydroxypyridine acts as an analogue of catechol but that the presence of a nitrogen atom in the ring alters its susceptibility to methylation.

Inhibition of AdoMet utilisation

The graphical methods (see Ref. 28) used to present the effect of 2,3-dihydroxypyridine inhibition on the utilisation of AdoMet by COMT, and the information derived from each plot are as follows (where v is initial velocity of reaction, s is substrate concentration and i is inhibitor concentration).

Dixon plot [23] (1/v *vs* i). The data did not respond well to this method yielding equivocal results which

Table 1. Purification of pig liver catechol-*O*-methyltransferase*

Stage	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Sp. act. (mU/mg protein)	Overall yield (%)	Overall purification (-fold)
Homogenate	2400	0.323	775.8	76.4	4.23	100	1.0
Supernatant	1840	0.381	700.8	68.5	5.56	90.3	1.31
Redissolved 55% saturating ammonium sulphate precipitate	128	2.35	301.2	137.3	17.14	38.8	4.0
Pooled Sephadex G75 fractions	650	0.608	395.1	10.3	58.85	50.9	13.9
Pooled affinity column fractions	50	2.27	113.4	1.04	2171.8	14.6	513.2

* See Ref. 16. Pig liver (967 g) was homogenised in 1500 ml 0.01 M triethanolamine hydrochloride buffer (pH 8) containing 0.13 M potassium chloride, 10 mM mercaptoethanol, 3 mM dithiothreitol, 0.2 mM phenylmethanesulphonyl fluoride, 2.6 mM magnesium chloride and 1 mM EDTA.
The catechol-*O*-methyltransferase assay contained (in a volume of 500 μl): 4 mM magnesium chloride, 3 mM catechol, 7.3 mM mercaptoethanol, 0.9 mM *S*-adenosyl-L-[^{3}H-methyl]methionine (0.497 Ci ^{3}H/mole), 0.64 units adenosine deaminase, 0.08 M potassium phosphate buffer (pH 7.6) and 5–123 μl appropriate fraction. Fifteen-minute incubation at 37°. See Ref. 18.

could be interpreted as mixed, competitive or uncompetitive inhibition. Other graphical methods were therefore employed.

Cornish-Bowden plot [29] [s/v *vs* i (*Fig.* 2)]. This procedure is acknowledged to be capable of determining inhibition constants not defined by the Dixon plot (see Ref. 28). An inhibition constant (K_i') of 85 μM (S.E.M. $\pm$ 0.09 μM, calculated as described earlier) was determined from this plot and either mixed or uncompetitive inhibition was indicated. The plot does not distinguish between these modes of inhibition.

Double-reciprocal plot (1/v *vs* 1/s). Results did not clearly favour any one mode of inhibition.

Hanes plot (s/v *vs* s). The data obtained were found to respond well to this treatment (Fig. 3), indicating an uncompetitive mode of inhibition by 2,3-dihydroxypyridine.

Woolf–Eadie–Hofstee plot (v *vs* v/s). Data responded moderately well to this treatment, indicating an uncompetitive mode of inhibition.

Thus, taken together, graphical evidence supports an uncompetitive mechanism against AdoMet, with a K_i' of 85 μM as determined by the Cornish-Bowden plot (Fig. 2). These findings agree with the work of Borchardt [15] who demonstrated competitive inhibition for structurally related pyridone and pyrone derivatives against the methyl acceptor substrate and an uncompetitive mode against AdoMet.

2,3-Dihydroxypyridine has been used as a reagent for the selective determination of iron in alloys [30] and is classified as an irritant. Pyridine is able to cause CNS depression and irritation of the skin and respiratory tract in man as well as being hepto- and nephrotoxic [31]. Chemical modification may be necessary to minimise the toxicity of 2-3-dihydroxypyridine toward other systems.

As a prototype inhibitor, 2,3-dihydroxypyridine has two advantages over catechol-derived inhibitors of COMT: (a) it is a "dead-end" inhibitor—not a substrate—and is not methylated by COMT; and (b) chemically, it is a more stable compound than catechol and is less likely to form toxic side-products. This stability should make the compound tractable during chemical modification designed to increase its specificity. Previous studies [Ref. 32 and Youde, Raxworthy and Gulliver (unpublished)] have indicated that increasing substrate hydrophobicity tends to cause tighter binding to COMT. Thus, such modifications could result in a very specific inhibitor derived from 2,3-dihydroxypyridine. It must be stressed, however, that 2,3-dihydroxypyridine is envisaged as a prototype inhibitor and no investigation into the compound's actions on tyrosine hydroxylase, DOPA decarboxylase or other enzyme systems has been undertaken in the present study.

Finally, the mechanism of the inhibition of COMT by 2,3-dihydroxypyridine may assist our understanding of the kinetic mechanism by which COMT interacts with its substrates. The simplest interpretation is that 2,3-dihydroxypyridine acts as a catechol analogue, but is not a substrate. It therefore competes with the catechol substrate for its binding site. However, the uncompetitive nature of the inhibition of 2,3-dihydroxypyridine against AdoMet can be most simply explained if COMT follows a

compulsory-order mechanism in which AdoMet binds first, followed by DOPAC.

Hence, 2,3-dihydroxypyridine binds to the enzyme–AdoMet complex. This agrees well with the work of Rivett and Roth [33] on membrane-bound COMT and reaches a consensus with some aspects of the double-displacement (substituted-enzyme) mechanism of Borchardt [34], but is less easy to reconcile with the random-order mechanism proposed by Flohé and Schwabe [35].

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