J. Enzyme Inhibition, 1996, Vol. 10, pp. 231–237
Reprints available directly from the publisher
Photocopying permitted by license only

SANGUINARINE AND CHELERYTHRINE AS INHIBITORS OF AROMATIC AMINO ACID DECARBOXYLASE

JAROSLAV DRŠATA,[1,*] JITKA ULRICHOVÁ[2] and DANIELA WALTEROVÁ[2]

[1]*Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic*
[2]*Institute of Medical Chemistry, Faculty of Medicine, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic*

(Received 11 December 1995; in final form 7 February 1996)

Quaternary benzo[c]phenanthridine alkaloids sanguinarine, chelerythrine and their dihydroderivatives were tested as inhibitors of aromatic amino acid decarboxylase (EC 4.1.1.28, AAD) from rat liver. Sanguinarine and chelerythrine exhibited strong inhibition of AAD with K_i 1.2×10^{-4} M and 5.8×10^{-4} M, respectively, while no inhibitory effect was observed for their dihydroderivatives. The inhibition was found to be irreversible. The enzyme-inhibitor interaction apparently stabilized AAD against thermal inactivation. Pyridoxal-5′-phosphate partially decreased but did not reverse the inhibition. Dithiothreitol prevented the inhibitory effect of sanguinarine and chelerythrine which indicates that the interaction with the thiol groups essential for AAD activity is included in the inhibition mechanism.

KEY WORDS: Aromatic amino acid decarboxylase, quaternary benzo[c]phenanthridine alkaloids, irreversible inhibition

INTRODUCTION

Aromatic L-amino acid decarboxylase (dopa decarboxylase, AAD; EC 4.1.1.28) catalyzes decarboxylation of aromatic amino acids to their respective biogenic amines.[1] Substrates with the highest affinity to AAD are 3,4-dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan. The enzyme thus plays a critical role in the biosynthesis of dopamine, noradrenaline, adrenaline and serotonin.[2] Other aromatic amino acids have lower affinity to the enzyme. AAD is widely distributed in mammalian tissues with high activities in the kidney and liver.[1] While AAD in neural tissues plays a neuron-specific role as a neurotransmitter biosynthetic enzyme, the enzyme in extra-neural tissues plays a presumably more general role as a nonspecific decarboxylating enzyme.[3]

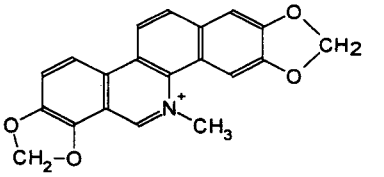
AAD is a pyridoxal-5′-phosphate-dependent enzyme; the coenzyme is bound to Lys-303 of the enzyme molecule,[4] forming a Schiff base with the amino group of the lysine side chain. The AAD molecule consists of two identical subunits with a subunit molecular weight of

*Correspondence.

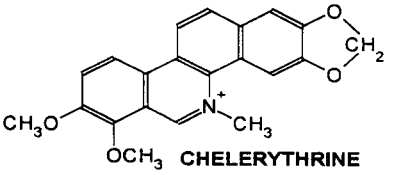
about 50 kDa. Each subunit of mammalian AAD contains 11–13 cysteine residues according to the species.[5] The thiol group of Cys-111, lying near the pyridoxal phosphate-binding site of AAD, is essential for the catalytic activity.[6]

A decreased synthesis of dopamine in the brain basal ganglia is one of the characteristic features of Parkinson's disease. Noradrenaline is a potent vasoconstrictor and serotonin as another AAD product belongs to inflammation mediators. That is why the compounds influencing AAD activity have been searched for.[7,8] Among these, α-hydrazino-α-methyldopa (carbidopa) and N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl) hydrazine (benserazide) are compounds that inhibit extracerebral AAD ("peripheral dopa decarboxylase") and thereby they enable simultaneously administered L-dopa to be exploited by the brain for dopamine production. Another important group of AAD inhibitors is represented by the α-chloro- and α-fluoromethyl derivatives of dopa, α-vinyldopa and α-acetylenic dopa, which act as enzyme-induced inactivators (mechanism-based or "suicide" inhibitors) and react with cysteine within the active centre of the enzyme molecule. Many AAD inhibitors are Schiff base analogues, coenzyme scavengers, organic acid derivatives, or amines showing various inhibition patterns.[7]

Benzophenanthridine alkaloids (BA) with an iminium bond ($C=N^+$) are strong electrophiles capable of interacting with nucleophilic sites in proteins.[9] Mainly SH-enzymes, e.g. aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2), Na^+K^+-ATPase (EC 3.6.1.37), Ca^{2+}-ATPase (EC 3.6.1.38), pyruvate dehydrogenase complex (EC 1.2.4.1, 2.3.1.12, 3.1.3.43),



SANGUINARINE



CHELERYTHRINE

are sensitive to BA.[10] Recently, inhibition of copper/quinoprotein amine oxidase (EC 1.4.3.6) by BA has been ascribed to the interaction with the enzyme thiol groups.[11] Generally, reduction of the iminium bond in the BA molecule leads to alteration of the biological activity of a parent alkaloid.[10]

The aim of this study is to investigate the inhibitory effect of BA — sanguinarine (SA), chelerythrine (CHE) and their dihydroderivatives on AAD activity.

MATERIALS AND METHODS

Materials

Rat liver was used as the source of AAD and the enzyme preparation was obtained as described previously.[8] The protein content was determined according to the method of Lowry.[12]

SA and CHE were isolated from an alkaloid extract of *Macleaya cordata* (*Papaveraceae*) (purchased from CAMAS Tech., Longmont, CO), both in a purity of approx. 96% (HPLC); dihydroderivatives of SA and CHE were obtained by reduction of BA with $NaBH_4$ in MeOH according to Brossi.[13]

Chemicals used in AAD assay: [1-^{14}C]-D,L-3-(3,4-dihydroxyphenyl)alanine (50 μCi.-mmol^{-1}, DuPont NEN, Germany), pyridoxal-5′-phosphate (Koch-Light Labs.), 1,4-dithiothreitol (DTT, Merck), Bray's scintillation coctail[14] (Spolana, Neratovice), dihydronicotinamide adenine dinucleotide, disodium salt (NADH, Merck). All chemicals used in the AAD assay were at least of analytical grade.

AAD assay and evaluation of inhibition

The enzyme activity was determined radiometrically.[15,16] The assay mixture contained AAD preparation, substrate [1-^{14}C]-dihydroxyphenylalanine at final concentrations of 2.5–12.5 $\times$ 10^{-4} M, pyridoxal-5′-phosphate (1×10^{-6} M usually), the alkaloid under study and other components at various concentrations depending on the aim of the individual experiments, in 0.02 M Na-phosphate buffer, pH 6.8. The AAD preparation was preincubated at 25°C with the alkaloid for 30 min before starting the enzyme assay unless stated otherwise. The assay mixture was then incubated under nitrogen at 37°C for 30 min and the radioactivity of $^{14}CO_2$ (liberated from the mixture by means of sulphuric acid and absorbed in 0.1 ml 30% KOH) was measured in the scintillation coctail[14] using a 1219 Rackbeta scintillation counter LKB Wallac. AAD activity was calculated on the basis of a known amount and specific radioactivity of $^{14}CO_2$ and expressed mostly in nkat per mg of protein. All data are the means ±S.D. of five measurements.

Estimation of K_i and inhibition kinetics

AAD inhibition by SA and CHE was studied in experiments with a constant concentration of AAD and variable concentration of the alkaloids at different concentrations of the substrate. The K_i value and type of inhibition were estimated graphically using Dixon ($1/v$ versus i) and Lineweaver and Burk ($1/v$ versus $1/s$) plots.

Reversibility of inhibition

After preincubation, the enzyme-alkaloid mixture was dialyzed against 0.02 M phosphate buffer, pH 6.8 at 4°C for 2 h with repeated exchange of the buffer. AAD activity of the mixture was then assayed. Control experiments were carried out in the absence of alkaloid. Alternatively, the reversibility of AAD inhibition was evaluated graphically using results of experiments with changing concentrations of the enzyme in the incubation mixture.[17]

Influence of the coenzyme on AAD inhibition

AAD activity was measured in the presence of the alkaloid (5×10^{-4} M) and in control samples after addition of different concentrations of pyridoxal-5′-phosphate, representing undersaturation (no external pyridoxal-5′-phosphate added) and saturation (1×10^{-6} and 1×10^{-5} M) of the enzyme with the coenzyme.[1]

Time profile of AAD inhibition

The alkaloid (1×10^{-4} M) was preincubated with the enzyme preparation for different time intervals, the assay mixture was then completed and the enzyme activity measured. Control samples without the alkaloid were processed in the same way. The same experiment was carried out in the presence of dithiothreitol.

Influence of Thiol protecting agent

Dithiothreitol (DTT, 1×10^{-4} or 1×10^{-5} M) was added to the enzyme preparation together with the alkaloid (1×10^{-4} or 5×10^{-4} M) and after preincubation AAD activity was assayed.

RESULTS AND DISCUSSION

Screening of BA and their dihydroderivatives suggested that SA (99% inhibition at 10^{-3} M concentration) and CHE (92% inhibition at 10^{-3} M concentration) were potent inhibitors of AAD under the conditions of the enzyme assay. Dihydroderivatives of SA and CHE did not exert any significant effect on activity of AAD up to 10^{-3} M concentration. The K_i values of AAD inhibition determined from the graphical evaluation of the experimental data were 1.2×10^{-4} M for SA, and 5.8×10^{-4} M for CHE, respectively. SA and CHE are thus relatively strong inhibitors of AAD.[7,8]

More detailed study of the inhibitor-enzyme interaction was further carried out with SA. Both Dixon and Lineweaver-Burk plots showed formally the noncompetitive inhibition type (Figure 1). Experiments with dialysis of the mixture of SA and the AAD preparation suggested that the inhibitor-enzyme interaction was completely irreversible. Due to the instability of the enzyme activity during storage and dialysis, partial loss of AAD activity was noted after dialysis even in the control samples (Table 1). In graphical evaluation of the reversibility of inhibition (Figure 2), parallelism of the lines for inhibited and control reaction seems to confirm the irreversibility of inhibition.[17]

TABLE 1
An attempt to restore AAD activity by dialysis of the enzyme-sanguinarine mixture.

	AAD activity (nkat/mg prot)	
Sample	*Before dialysis*	*After dialysis*
Control	180.2±5.9	110.6±2.8
+ Sanguinarine	94.5±1.9(52)*	47.5±4.0(43)*

*Percent of control activity.

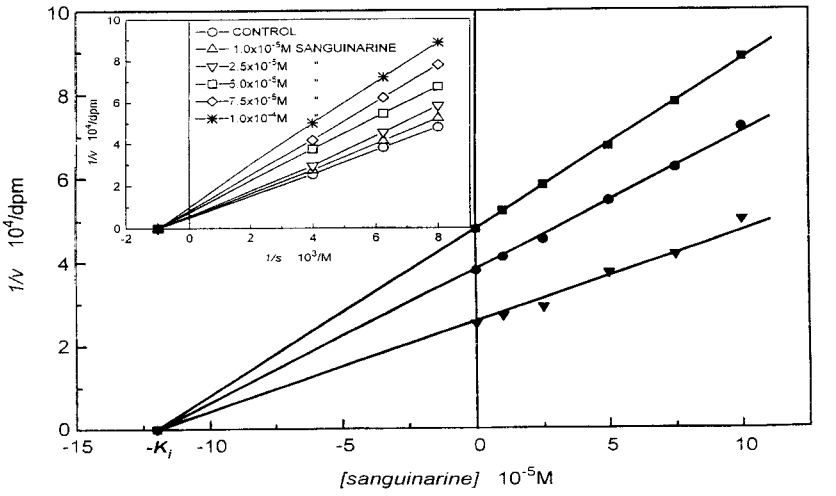


FIGURE 1 Estimation of K_i and type of AAD inhibition by sanguinarine. The Dixon plot is presented in the main graph; the insert shows the Lineweaver-Burk plot based on the same data. The assay was carried out with 266 pkat of the enzyme. Substrate concentrations in the main graph: 1.25■, 2.00● and 2.5 × 10^{-4} M▼.

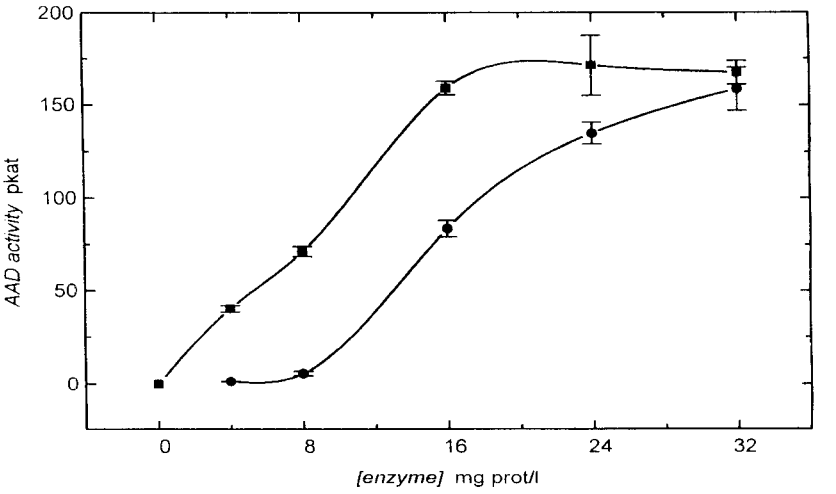


FIGURE 2 AAD activity and AAD inhibition as a function of the enzyme concentration. ■ Control activities. • Sanguinarine 5 × 10^{-4} M.

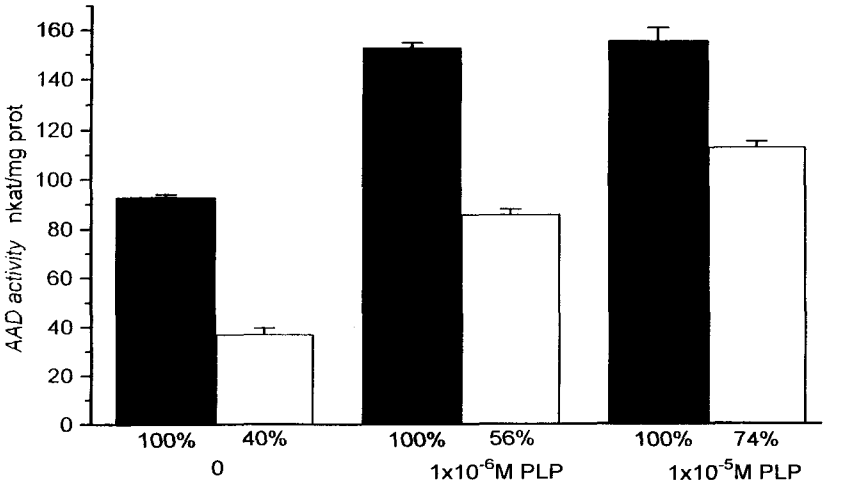


FIGURE 3 Influence of the addition of pyridoxal-5′-phosphate (PLP) on AAD inhibition by sanguinarine. ■ = control AAD activities. □ = sanguinarine 5×10^{-4} M.

Experiments with the addition of an external coenzyme to the incubation mixture confirmed the experience that AAD activity *in vitro*, in spite of the presence of tightly bound pyridoxal-5′-phosphate in the enzyme molecule, can be stimulated by the external coenzyme.[1,15] Inhibition of AAD by SA was partially reduced when the coenzyme was added (Figure 3). This suggests some difference in the affinity of the alkaloid to the coenzyme-saturated and coenzyme-unsaturated molecules of AAD.

AAD inhibition by SA (5×10^{-4} M) was completely prevented by DTT at a concentration of 10^{-3} M. The time-dependence of this effect was further studied together with the time profile of AAD inhibition by SA. The time course of AAD inhibition by SA was remarkable. During preincubation of SA with the enzyme preparation at 25°C, the alkaloid gradually showed some kind of protection of AAD activity against thermal inactivation, so that after 24 h the mixture of AAD with SA had higher catalytic activity than the enzyme alone. This effect was DTT-independent, though DTT itself increased control AAD activity as an efficient thiol protector (Figure 4). DTT at the same concentration as SA (1×10^{-4} M) reduced AAD inhibition from 42% to only 10–5% between 0 and 10 h of the experiment (Figure 4). These results suggest that in AAD inhibition by SA and its congeners, as in the case of inhibition of several other enzymes,[10,11] the interaction of the compound iminium bond with the thiol group essential for the enzyme activity plays an important role. This is consistent with the finding that dihydrobenzophenanthridines lacking the iminium bond do not inhibit AAD activity.

Acknowledgements

This work was supported by the grant 303/93/527 from the Grant Agency of the Czech Republic. We thank Prof. M. Lázníček (Radioisotope Laboratory, Faculty of Pharmacy, Charles University, Hradec Králové) for measurement of samples on a liquid scintillation counter and Mrs. B. Navrátilová for technical assistance.

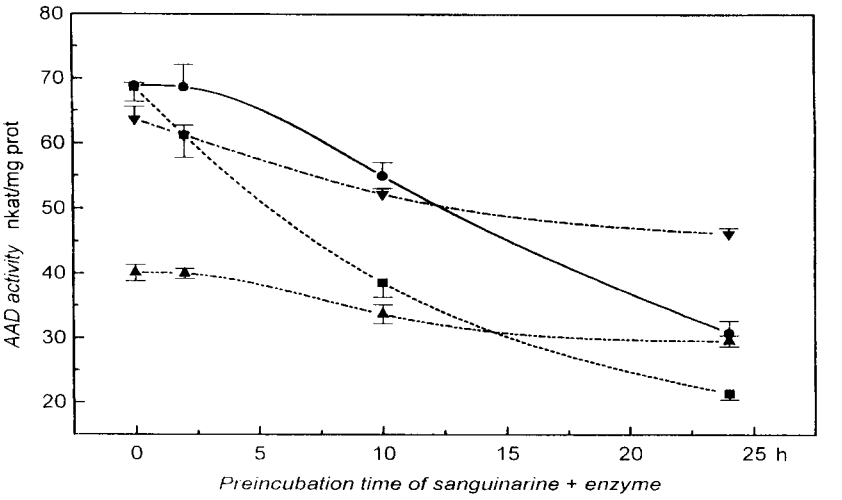


FIGURE 4 Time profile for AAD inhibition by sanguinarine and influence of dithiothreitol (DTT). Intervals of preincubation presented on the abscissa do not include an obligatory 30 min preincubation before commencing the assay. ■ Control AAD activity. • DTT 1×10^{-4} M. ▲ Sanguinarine 1×10^{-4} M. ▼ Sanguinarine 1×10^{-4} M + DTT 1×10^{-4} M.

References

1. Christenson, J.G., Dairman, W. and Udenfriend, S. (1970) *Arch. Biochem. Biophys.*, **141**, 356–357.
2. Udenfriend, S. (1964) *Vitam. Horm.*, **22**, 445–450.
3. Dominici, P., Filipponi, P., Schinina, M.E., Barra, D. and Borri Voltatorni, C. (1990) *Ann. N.Y. Acad. Sci.*, **585**, 162–172.
4. Borri Voltatorni, C., Minelli, A., Vecchini, P., Fiori, A. and Turano, C. (1979) *Eur. J. Biochem.*, **93**, 181–188.
5. Nagatsu, T. (1991) *Neurosci. Res.*, **12**, 315–345.
6. Dominici, P., Maras, B., Mei, G. and Borri Voltatorni, C. (1991) *Eur. J. Biochem.*, **201**, 393–397.
7. Jung, M.J. (1986) *Bioorg. Chem.*, **14**, 429–443.
8. Přibová, M., Gregorová, J. and Držata, J. (1992) *Pharmacol. Res.*, **25**, 271–277.
9. Šimánek, V. (1985) In *The Alkaloids*, (Brossi, A., ed.), Vol. **26**, pp. 185–240. Academic Press: Orlando.
10. Walterová, D., Ulrichová, J., Válka, I., Vičar, J., Vavrečková, C., Táborská, E., Harkrader, R.J., Meyer, D.L., Černá, H. and Šimánek, V. (1995) *Acta Univ. Palacki. Olomuc., Fac. Med.*, **139**, 5–22.
11. Luhová, L., Frébort, I., Ulrichová, J., Adachi, O., Šimánek, V. and Peč, P. (1995) *J. Enzym. Inhib.*, **9**, 295–302.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.*, **193**, 265–275.
13. Brossi, A. and Borrer, R. (1965) *Lloydia*, **28**, 199–202.
14. Bray, G.A. (1960) *Anal. Biochem.*, **1**, 279–285.
15. Držata, J. and Hais, I.M. (1982) *Folia Pharm. Univ. Carol.*, **3**, 69–88.
16. Držata, J. and Hais, I.M. (1973) *Chem. Listy*, **67**, 853–859.
17. Segel, I.H. (1975) *Enzyme Kinetics*, p. 125. Wiley and Sons; New York.