J. Enzyme Inhibition, 1998, Vol. 13, pp. 465–471
Reprints available directly from the publisher
Photocopying permitted by license only

COMPETITIVE INHIBITION OF *LENS CULINARIS* L. COPPER AMINE OXIDASE BY AMILORIDE, *p*-AMINOBENZAMIDINE, CLONIDINE, 4′,6-DIAMIDINO-2-PHENYLINDOLE AND GABEXATE MESYLATE: A COMPARATIVE STUDY

LUCA ERCOLINI, RICCARDO ANGELINI, RODOLFO FEDERICO, GIUSEPPINA REA, GIORGIO VENTURINI and PAOLO ASCENZI*

Department of Biology, Third University of Rome, Viale Guglielmo Marconi 446, 00146 Rome, Italy

(Received 12 February 1998; In final form 25 March 1998)

The competitive inhibition of *Lens culinaris* L. copper amine oxidase by amiloride ($K_i = 4.1 \times 10^{-4}$ M), *p*-aminobenzamidine ($K_i = 6.0 \times 10^{-4}$ M), clonidine ($K_i = 5.0 \times 10^{-4}$ M), 4′,6-diamidino-2-phenylindole (DAPI; $K_i = 1.9 \times 10^{-5}$ M) and gabexate mesylate ($K_i = 2.5 \times 10^{-4}$ M) has been investigated, at pH 7.0 and 25°C. The affinity of *p*-aminobenzamidine, clonidine and DAPI for plant and mammalian copper amine oxidase is closely similar. However, values of K_i for amiloride and gabexate mesylate binding to swine kidney copper amine oxidase are lower than those observed for inhibitor binding to *Lens culinaris* L. copper amine oxidase. Thus, amiloride and gabexate mesylate may represent useful model compounds for the development of selective inhibitors of mammalian copper amine oxidase, which may be important in view of the potential use of plant copper amine oxidase as drugs.

Keywords: *Lens culinaris* L. copper amine oxidase; Amiloride; *p*-Aminobenzamidine; Clonidine; 4′,6-Diamidino-2-phenylindole; Gabexate mesylate; Competitive enzyme inhibition

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole

* Corresponding author. Tel.: +39+6+55176319. Fax: +39+6+55176321.
E-mail: ascenzi@bio.uniroma3.it.

INTRODUCTION

Copper amine oxidase (E.C. 1.4.3.6) is a heterogeneous group of enzymes which catalyse the oxidative deamination of various biogenic amines, including mono-, di- and poly-amines, such as histamine, putrescine and spermidine, with the production of the corresponding aldehydes, ammonium, and hydrogen peroxide.[1,2] These enzymes are homodimers of 70–90 kDa subunits, each containing a single copper ion and a covalently bound cofactor formed by the post-translational modification of a tyrosine residue to 2,4,5-trihydroxyphenylalanine quinone.[1,3] In plants, copper amine oxidase has been hypothesised to have a role in the production of hydrogen peroxide essential in peroxidase-mediated lignification and cross-linking of extracellular macromolecules, both during ontogenesis and as a response in wound-healing and defence toward pathogens.[4,5] In mammals, copper amine oxidase activity is highest in the small intestine and maternal placenta where it may have a protective role towards elevated levels of amine present.[6]

Mammalian copper amine oxidase, administrated in rats with Erlich ascite tumours, inhibits tumour cell growth, possibly through the cytotoxic effect of its reaction products (i.e. aminoaldehydes and hydrogen peroxide).[7] Since plant copper amine oxidase shows the highest specific activity, and the highest rate of reoxidation of the reduced enzyme,[1] it may be more effective than the mammalian enzyme. The potential use of plant copper amine oxidases as drugs raises the requirement for the development of new specific inhibitors which should discriminate between the endogenous and exogenous enzyme in such studies.

Present results indicate that amiloride, *p*-aminobenzamidine, clonidine, 4′,6-diamidino-2-phenylindole (DAPI) and gabexate mesylate are competitive inhibitors of *L. culinaris* L. copper amine oxidase. The affinity of amiloride and gabexate mesylate for swine kidney copper amine oxidase is higher than that observed for inhibitor binding to *Lens culinaris* L. copper amine oxidase. Thus, amiloride and gabexate mesylate may represent useful model compounds for the development of selective inhibitors of mammalian copper amine oxidase, and may be important in view of the potential use of plant copper amine oxidases as drugs.

MATERIALS AND METHODS

Lens culinaris L. copper amine oxidase was purified as detailed elsewhere.[8] Swine kidney copper amine oxidase was kindly provided by Prof. B. Mondovì

(Department of Biochemical Sciences 'A. Rossi Fanelli', University of Rome 'La Sapienza', Rome, Italy).

Agmatine, amiloride, *p*-aminobenzamidine, clonidine, DAPI, putrescine and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Gabexate mesylate was purchased from Lepetit S.p.A. (Milano, Italy). All the other products were obtained from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and were used without further purification.

Oxidation of agmatine and putrescine catalysed by *Lens culinaris* L. and swine kidney copper amine oxidase was investigated in the absence and presence of amiloride, *p*-aminobenzamidine, clonidine, DAPI and gabexate mesylate, as previously reported.[2,9] In the enzyme assay, the copper amine oxidase concentration ranged between 2.0×10^{-10} and 5.0×10^{-10} M, the inhibitor concentration between 1.0×10^{-5} and 8.0×10^{-4} M, and the substrate concentration between 1.0×10^{-5} and 2.0×10^{-4} M. In a typical experiment: 20 μL of a buffered *Lens culinaris* L. or swine kidney copper amine oxidase solution was added to 1.0 mL of a buffered solution containing the substrate and/or the inhibitor. The initial velocity for the enzymatic oxidation of agmatine and putrescine was then measured. The initial velocity was unaffected by the enzyme/inhibitor incubation time, preceding substrate addition, and by the enzyme/substrate incubation time, preceding inhibitor addition. In fact, the enzyme/inhibitor/substrate equilibration time was very short, being complete in the mixing time (about 5 s).

Values of K_i for *Lens culinaris* L. copper amine oxidase competitive inhibition by amiloride, *p*-aminobenzamidine, clonidine, DAPI and gabexate mesylate were determined from the linear dependence of the K_m^{app}/K_m^0 ratio on the inhibitor concentration ([I]), according to

$$K_m^{app}/K_m^0 = K_i^{-1} \cdot [I] + 1, \quad (1)$$

where K_m^{app} is the apparent Michaelis constant determined at a fixed inhibitor concentration, and K_m^0 is the intrinsic Michaelis constant determined in the absence of the inhibitor.[10] Values of K_i for swine kidney amine oxidase competitive inhibition by *p*-aminobenzamidine and DAPI were also determined, according to Eq. (1).[10] Values of K_i for swine kidney amine oxidase competitive inhibition by amiloride, clonidine and gabexate mesylate were obtained from the literature.[2] Values of K_m^0 and K_m^{app} for agmatine and putrescine oxidation catalysed by *Lens culinaris* L. and swine kidney copper amine oxidase were determined from the dependence of the initial velocity on the substrate concentration, in the absence and presence of the inhibitor,

respectively. Kinetics was analysed according to the Michaelis–Menten equation.[10] Values of K_m^0 for agmatine and putrescine oxidation catalysed by *Lens culinaris* L. copper amine oxidase were 1.2×10^{-4} and 5.0×10^{-5} M, respectively. Values of K_m^0 for agmatine and putrescine oxidation catalysed by swine kidney copper amine oxidase were 1.0×10^{-5} and 3.0×10^{-5} M, respectively. As expected for a simple competitive inhibition system,[10] values of V_{max} were unaffected by the inhibitor, and values of K_i were independent of the substrate, e.g. agmatine and putrescine, within the experimental error (± 5.0%). All data were obtained at pH 7.0 (1.0×10^{-1} M phosphate or Hepes buffer), and 25°C. Values of K_i were unaffected by the buffer system used.

RESULTS AND DISCUSSION

Chemical structures for amiloride, *p*-aminobenzamidine, clonidine, DAPI and gabexate mesylate are given in Figure 1.

As shown in Figure 2 and Table I, amiloride, *p*-aminobenzamidine, clonidine, DAPI and gabexate mesylate competitively inhibit *Lens culinaris* L. and swine kidney copper amine oxidase. The value of K_i for swine kidney copper amine oxidase inhibition by DAPI given in Table I ($= 1.6 \times 10^{-5}$ M) is close to that reported in the literature ($= 1.3 \times 10^{-5}$ M; pH 7.5, 1.0×10^{-1} M phosphate buffer, and 37°C).[11] Moreover, amiloride inhibits human placental copper amine oxidase with $K_i = 5.1 \times 10^{-6}$ M (pH 8.2, 2.0×10^{-2} M Tris/HCl buffer, and 37°C).[12] The affinity of *p*-aminobenzamidine, clonidine and DAPI for plant and mammalian copper amine oxidase is closely similar (see Table I). On the other hand, values of K_i for amiloride and gabexate mesylate binding to swine kidney copper amine oxidase, and for amiloride binding to human placental copper amine oxidase[12] are lower than those observed for inhibitor binding to *Lens culinaris* L. copper amine oxidase (see Table I). Thus, amiloride and gabexate mesylate may represent useful model compounds for the development of selective inhibitors of mammalian copper amine oxidase. Inspection of the three-dimensional structures of homologous copper amine oxidase from *E. coli*[13,14] and from *Pisum sativum* L.[15] suggests a non-covalent reversible electrostatic interaction(s) between the inhibitor and the enzyme 2,4,5-trihydroxyphenyl-alanine quinone-Asp catalytic diad.[2]

Agmatine and clonidine derivatives, amiloride, *p*-aminobenzamidine, DAPI and gabexate mesylate inhibit competitively trypsin-like serine proteinases.[16–19] Moreover, agmatine, amiloride and *p*-aminobenzamidine

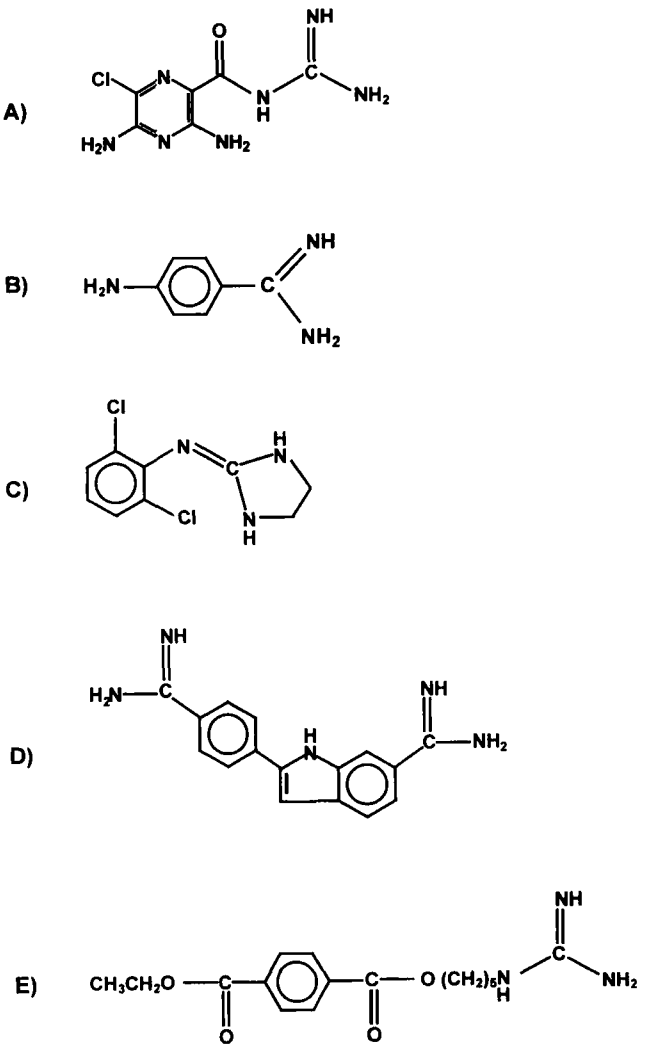

FIGURE 1 Chemical structures of amiloride (A), *p*-aminobenzamidine (B), clonidine (C), DAPI (D) and gabexate mesylate (E).

inactivate competitively nitric oxide synthase.[20–22] However, these chemicals are not nitric oxide precursors.[20–22] DAPI also inhibits competitively S-adenosyl-L-methionine decarboxylase.[11] Moreover, these compounds might also affect arginase, L-arginine-glycine transaminase, kyotorphine synthase and L-arginine decarboxylase, all using L-arginine as the

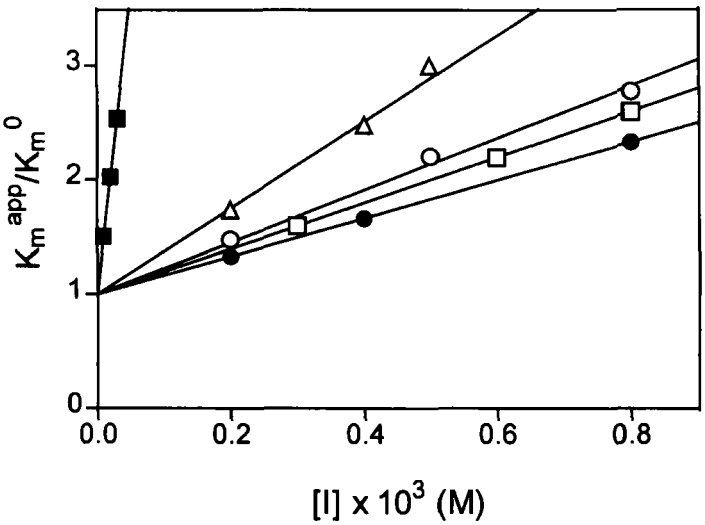

FIGURE 2 Effect of amiloride (○), *p*-aminobenzamidine (●), clonidine (□), DAPI (■) and gabexate mesylate (Δ) concentrations (i.e. [I]) on the K_m^{app}/K_m^0 ratio for the competitive inhibition of the *Lens culinaris* L. copper amine oxidase catalysed oxidation of agmatine and putrescine. Values of K_m^0 for the *Lens culinaris* L. copper amine oxidase catalysed oxidation of agmatine and putrescine are 1.2×10^{-4} and 5.0×10^{-5} M, respectively. The continuous lines were calculated according to Eq. (1) and the K_i values are given in Table I. All data were obtained at pH 7.0 (1.0×10^{-1} M phosphate or Hepes buffer), and 25°C. For further details, see text.

TABLE 1 K_i values for amiloride, *p*-aminobenzamidine, clonidine, DAPI and gabexate mesylate binding to *Lens culinaris* L. and swine kidney copper amine oxidase

Inhibitor	K_i (M)	
	Lens culinaris L. *copper amine oxidase*[a]	*Swine kidney copper amine oxidase*
Amiloride	4.1×10^{-4}	1.0×10^{-5} [b]
p-Aminobenzamidine	6.0×10^{-4}	2.5×10^{-4} [a]
Clonidine	5.0×10^{-4}	9.0×10^{-4} [b]
DAPI	1.9×10^{-5}	1.6×10^{-5} [a]
Gabexate mesylate	2.5×10^{-4}	2.7×10^{-5} [b]

[a] pH 7.0 (1.0×10^{-1} M phosphate or Hepes buffer), and 25°C. Present study.
[b] pH 7.0 (1.0×10^{-1} M phosphate buffer), and 25°C.[2]

substrate.[23] Thus, the use of amiloride, clonidine and gabexate mesylate as drugs should be under careful control. The knowledge of the selective inhibition mechanisms of these L-arginine related compounds could be a tool in designing pharmacologically active recombinant copper amine oxidase.

Acknowledgments

Authors thank Prof. E. Menegatti, Dr. M. Colasanti and Dr. A. Mattevi for helpful discussions. This study was partially supported by grants from the Ministry of University, Scientific Research and Technology of Italy (MURST Target Project on 'Biocatalysis and Bioconversion'), and of the National Research Council of Italy (CNR Target Project on 'Biotechnology').

References

[1] McIntire, W.S. and Hartmann, C. (1984) In *Principles and Applications of Quinoproteins*, (Davison, V.L., ed.), pp. 97–171. Marcel Dekker; New York.
[2] Federico, R., Angelini, R., Ercolini, L., Venturini, G., Mattevi, A. and Ascenzi, P. (1997) *Biochim. Biophys. Res. Commun.*, **240**, 150–152.
[3] Klinman, J.P. (1996) *Chem. Rev.*, **96**, 2541–2561.
[4] Angelini, R., Manes, F. and Federico, R. (1990) *Planta*, **182**, 89–96.
[5] Angelini, R., Bragaloni, M., Federico, R., Infantino, A. and Porta-Puglia, A. (1993) *J. Plant Physiol.*, **142**, 704–709.
[6] Sessa, A. and Perin, A. (1994) *Agents and Actions*, **43**, 69–77.
[7] Agostinelli, E., Przybytkowski, E., Mondovì, B. and Averill-Bates, D. (1994) *Biochem. Pharmacol.*, **48**, 1181–1186.
[8] Floris, G., Giartosio, A. and Rinaldi, A. (1983) *Phytochemistry*, **22**, 1871–1874.
[9] Angelini, R., Rea, G., Federico, R. and D'Ovidio, R. (1996) *Plant Sci.*, **119**, 103–113.
[10] Ascenzi, P., Ascenzi, G.M. and Amiconi, G. (1987) *Biochem. Educ.*, **15**, 134–135.
[11] Cubria, C., Alvarez-Bujidos, M., Negro, A., Balaña-Fouce, R. and Ordóñez, D. (1993) *Comp. Biochem. Physiol.*, **C 105**, 251–254.
[12] Novotny, W.F., Chassande, O., Baker, M., Lazdunski, M. and Barbry, P. (1994) *J. Biol. Chem.*, **269**, 9921–9925.
[13] Parsons, M.R., Convery, M.A., Wilmot, C.M., Yadav, K.D.S., Blakeley, V., Corner, A.S., Phillips, S.E.V., McPherson, M.J. and Knowles, P.F. (1995) *Structure*, **3**, 1171–1184.
[14] Wilmot, C.M., Murray, J.M., Alton, G., Parsons, M.R., Convery, M.A., Blakeley, V., Corner, A.S., Palcic, M.M., Knowles, P.F., McPherson, M.J. and Phillips, S.E.V. (1997) *Biochemistry*, **36**, 1608–1620.
[15] Kumar, V., Dooley, D.M., Freeman, H.C., Mitchell Guss, J., Harvey, I., McGuirl, M., Wilce, M.C.J. and Zubak, V.M. (1996) *Structure*, **4**, 943–955.
[16] Barrett, A.J. and Salvesen, G. (eds.) (1986) *Proteinase Inhibitors*. Elsevier; Amsterdam, New York and Oxford.
[17] Menegatti, E., Bolognesi, M., Scalia, S., Bortolotti, F., Guarneri, M. and Ascenzi, P. (1986) *J. Pharm. Sci.*, **75**, 1171–1174.
[18] Vassalli, J.-D. and Belin, D. (1987) *FEBS Lett.*, **214**, 187–191.
[19] Casale, E., Collyer, C., Ascenzi, P., Balliano, G., Milla, P., Viola, F., Fasano, M., Menegatti, E. and Bolognesi, M. (1995) *Biophys. Chem.*, **54**, 75–81.
[20] Galea, E., Regunathan, S., Eliopoulos, V., Feinstein, D.L. and Reis, D.J. (1996) *Biochem. J.*, **316**, 247–249.
[21] Ascenzi, P., Federico, R., Menegatti, E. and Venturini, G. (1997) *Biochem. Mol. Biol. Int.*, **43**, 507–511.
[22] Venturini, G., Menegatti, E. and Ascenzi, P. (1997) *Biochem. Biophys. Res. Commun.*, **232**, 88–90.
[23] Nakaki, T. and Kato, R. (1994) *Jpn. J. Pharmacol.*, **66**, 167–171.