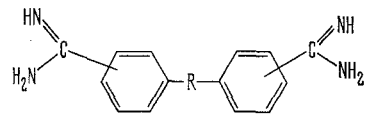
Inhibitory Effect of Aromatic Diamidines on Trypsin and Enterokinase

The search for ever stronger inhibitors of trypsin and enterokinase led within a few years from rather weak aliphatic amino and guanidino compounds to the highly effective derivatives of benzamidine[1-9]. The most powerful monocyclic aromatic compound was found in *p*-amidinophenylpyruvic acid which also turned out to be an excellent inhibitor of thrombin, plasmin and kallikrein[10-12]. Lately, attempts to further improve on the strength of the inhibitors have been directed towards benzamidine derivatives bearing a second phenyl residue. In the compounds examined so far the 2 benzene rings have been connected by either an ester linkage or by an α-ω-dioxyalkane bridge[11,13-16].

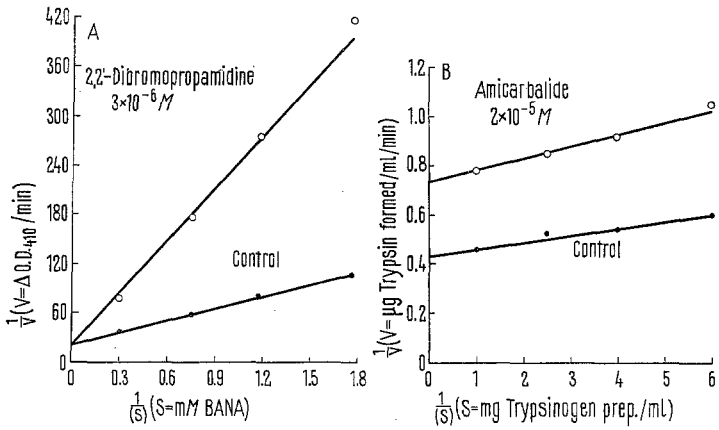
The departure point for the investigation reported here was the previous observation that presence of 2 amidino groups in *para* position on a single benzene ring (1,4-diamidinobenzene) imparted only a moderate degree of trypsin-inhibitory power, while presence of an amidino group in *para* position on each of 2 linked benzene rings (4,4'-diamidinodiphenylamine) led to an inhibitor even stronger than *p*-aminobenzamidine[10]. Additional diphenyl diamidines have now been examined for their inhibitory activity against trypsin and enterokinase, and all of them were found to be markedly effective. The results are of considerable interest because all the diamidines newly tested in this study are being used in the treatment of certain protozoal and fungal diseases and because the mechanism of action of the drugs has never been satisfactorily explained. In the light of the data reported below one wonders whether the pharmacological effect could not be due to inhibition of proteases in the parasites. By necessity, these hypothetical enzymes would have to share specificity characteristics with trypsin and enterokinase.

Materials and methods. Bovine trypsin (2× crystallized, salt-free) and bovine trypsinogen (1× crystallized; 50% by weight proenzyme, 50% $MgSO_4$) were obtained from Mann Research Laboratories, Inc. Porcine enterokinase (0.53 E.K.U./mg, trypsin-free) originated from Calbiochem. *p*-Aminobenzamidine-2HCl was supplied by Cyclo Chemical, Division of Travenol Laboratories, Inc. 4,4'-Diamidinodiphenylamine-2HCl was purchased from K & K Laboratories. All other diamidines were products of May & Baker Ltd. Their mode of synthesis and their chemical and physical characteristics have been described[17-21], and they share this basic structural formula:



The compounds were the following: stilbamidine isethionate (4,4'-stilbenedicarboxamidine diisethionate), 2-hydroxystilbamidine isethionate, propamidine isethionate (*p,p'*-(trimethylenedioxy)di-benzamidine *bis* (β-hydroxyethanesulfonate)), 2,2'-dibromopropamidine isethionate, pentamidine isethionate (*p,p'*-(pentamethylenedioxy)di-benzamidine *bis* (β-hydroxyethanesulfonate)), amicarbalide (3,3'-diamidinocarbanilide diisethionate), and M & B 4596 (2,7-di(m-amidinophenyldiazoamino)-10-ethyl-9-phenyl-phenanthridinium chloride di HCl).

The activity of trypsin was assayed by using N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BANA) as substrate[22]. The reaction mixtures consisted of 3.2 ml of 0.08 *M* *Tris*-HCl buffer (pH 8.1) containing 5 µg trypsin, 0.02 *M* $CaCl_2$, various concentrations of inhibitor and 5.7×10^{-4} *M*



Double reciprocal plots according to LINEWEAVER and BURK[24] demonstrating that 2,2'-dibromopropamidine is a competitive inhibitor of the hydrolysis of BANA by trypsin (A), and that amicarbalide inhibits enterokinase in a noncompetitive manner (B).

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to $3.4 \times 10^{-3} M$ BANA. Incubation was carried out for 15 min at 37 °C. Enterokinase was determined by its ability to activate trypsinogen. The activation mixtures of 2 ml of $0.02 M$ phosphate buffer (pH 5.8) contained 0.5 mg enterokinase, 0.33–2.0 mg trypsinogen and various amounts of inhibitor. Activation was allowed to proceed for 20 min at 25 °C. The trypsin formed was measured with BANA as substrate. The inhibition constants (K_i) for the amidino compounds were obtained graphically by plotting 1/v against inhibitor concentration at 2 or 3 different substrate concentrations[23]. While these graphs were already indicative of the competitive or noncompetitive nature of the inhibition, confirmation of the type of inhibition was always sought from data arranged in a double reciprocal plot according to LINEWEAVER and BURK[24].

Results and discussion. From the Table it is evident that all new diamidines examined were stronger trypsin inhibitors than either *p*-aminobenzamidine or 4,4′-diamidinodiphenylamine, both of which were included in the study for comparison. The last 5 compounds listed also outranked *p*-amidinophenylpyruvic acid, the K_i value of which with BANA has been reported as $6.0 \times 10^{-6} M$[11]. Amicarbalide and M & B 4596, the only 2 compounds with the amidino groups in the *meta* position, possessed considerable activity against enterokinase but they were not able to match the strength of *p*-amidinophenylpyruvic acid ($K_i = 9.7 \times 10^{-7} M$)[10]. It should be noted that, overall, the inhibitors showed no parallelism between their activity against trypsin on the one hand, and against enterokinase on the other hand. Kinetically, all amidines inhibited BANA hydrolysis by trypsin in a competitive fashion, while against enterokinase only a noncompetitive type of inhibition was encountered. Representative kinetic graphs are demonstrated in the Figure.

The best trypsin inhibitor in the series here, 2,2′-dibromopropamidine, appears to possess about 60% of the activity of the strongest low-molecular weight compound recorded to date, i.e., 4-guanidino-benzoic acid 4′-nitrobenzyl ester[25]. As a reversible inhibitor 2,2′-dibromopropamidine is about equal in potency to the most effective inhibitors produced by BAKER and ERICKSON[15,16] which, like 2,2′-dibromopropamidine, are also phenoxyalkoxy derivatives of benzamidine. Most of their compounds, however, are reversible inhibitors only early during the reaction with trypsin while later on they become covalently bound to the enzyme due to the presence of a fluorosulfonylbenzamide moiety on the second phenyl ring[26].

Inhibition of trypsin and enterokinase by aromatic amidines

Compound	Trypsin K_i (M)	Enterokinase
p-Aminobenzamidine	1.7×10^{-5}	2.0×10^{-5}
4,4′-Diamidinodiphenylamine	6.4×10^{-6}	2.3×10^{-4}
2-Hydroxystilbamidine	6.2×10^{-6}	3.8×10^{-4}
Stilbamidine	6.0×10^{-6}	1.2×10^{-3}
Amicarbalide	4.6×10^{-6}	3.0×10^{-5}
Propamidine	3.3×10^{-6}	1.3×10^{-3}
Pentamidine	2.3×10^{-6}	9.5×10^{-4}
M & B 4596	1.9×10^{-6}	1.1×10^{-5}
2,2′-Dibromopropamidine	7.5×10^{-7}	5.0×10^{-4}

Zusammenfassung. Aromatische Diamidinoverbindungen sind hochwirksame Hemmstoffe des Trypsins. 2,2′-Dibromopropamidin, die stärkste der hier untersuchten Verbindungen, besitzt gegenüber der BANA-Hydrolyse eine Inhibitions-Konstante von $7.6 \times 10^{-7} M$. Amicarbalid und M & B 4596 sind gute Hemmstoffe der Enterokinase, doch erreichen sie die Wirkung der *p*-Amidinophenylbrenztraubensäure nicht.

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[26] The author is grateful to May & Baker Ltd. for the gift of the aromatic diamidines. This study was supported by U.S. Public Health Service grants No. AM 10746 and HE 6350.

Oxidative Activity of Limbic Structures During Sexual Cycle in Rats[1]

It has been demonstrated that the oxygen consumption of hypothalamus undergoes modifications during the sexual cycle in rats[2,3]; lower respiratory rates were observed during diestrus and higher ones during estrus. Apparently the hypothalamic metabolic changes are connected with the mechanisms that control the ovulation and with the sexual hormones that induce the estrus phase[4].

Remembering that the hypothalamus is in relation with the limbic structures and that such structures participate in some way in the ovulatory process[5], it seems to be of interest to determine the oxidative metabolism of the hippocampus and amygdala in female rats during the sexual cycle.

Material and methods. Wistar female rats were used. The animals were fed on the standard diet of the Catedra de Fisiología. Vaginal smears were performed before sacrifice. The animals were decapitated and the amygdala, hippocampus and a sample of cerebral cortex removed. Oxygen uptake was determined by Warburg manometry in vessels of 12–15 ml capacity, containing 3 ml of Krebs-Ringer phosphate buffer, pH 7.4 and 7.7 mM glucose; the central well contained 0.2 ml of saturated

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