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INHIBITION OF GUANIDINOBENZOATASE BY A SUBSTRATE FOR TRYPSIN-LIKE ENZYMES

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Guanidinobenzoatase is a proteolytic enzyme capable of degrading fibronectin and is a tumour associated enzyme. Guanidinobenzoatase has been shown to be an arginine selective protease and is distinct from trypsin, plasmin and thrombin, the latter enzymes can be assayed with bis(carbobenzyloxycarbonyl-L-argininamido)-Rhodamine or BZAR. Guanidinobenzoatase is inhibited by BZAR when the enzyme is assayed in free solution and when the enzyme is cell-bound in frozen sections of tumour containing tissues. It is proposed that BZAR and its analogues may be of value in inhibiting tumour cell invasion *in vivo* and also that the selectivity of BZAR may be used to direct cytotoxic drugs to tumour cells possessing active guanidinobenzoatase.

KEY WORDS: Guanidinobenzoatase, Tumour protease, Selective inhibition.

INTRODUCTION

Guanidinobenzoatase is a trypsin-like enzyme[1,2] associated with tumour cells and cells capable of migration, such as infiltrating lymphocytes.[2,3] The enzyme acts on 4-nitrophenyl-*p*-guanidinobenzoate and 4-methylumbelliferyl-*p*-guanidinobenzoate as true substrates[1,2] rather than active site titrants,[4,5] whereas these compounds are suicide substrates for trypsin, thrombin and plasmin. Guanidinobenzoatase degrades fibronectin and the peptide GlyArgGlyAsp linking cells to fibronectin.[6] Active guanidinobenzoatase on the surface of tumour cells may be located by the fluorescent probe, 9-aminoacridine, a competitive inhibitor of guanidinobenzoatase.[2,7] We wish to draw attention to the observation that a fluorogenic substrate for trypsin-like enzymes (Cbz-Arg-NH)$_2$-Rhodamine or BZAR[8] is a good inhibitor of guanidinobenzoatase both in solution and on the surface of tumour cells. It is known that single tumour cells capable of metastasis possess uninhibited guanidinobenzoatase[9] and as

a consequence compounds with the selective inhibitory properties of BZAR are of potential interest in the control of tumour spread.

MATERIALS AND METHODS

9-Aminoacridine and 4-methylumbelliferyl-*p*-guanidinobenzoate were purchased from Sigma Chemical Company, St. Louis, Mo, U.S.A. BZAR was provided by WFM and prepared according to the method previously reported.[8] Frozen sections of tumour-containing tissues were kindly provided by the Department of Pathology, Justus-Liebig University, Giessen, West Germany. Mouse Ehrlich ascitic plasma was prepared by Dr. S. Itzhaki of this department and was used as the source of crude guanidinobenzoatase in the kinetic analysis of inhibition by BZAR.

The assay of guanidinobenzoatase[1] employed 10 μl mouse Ehrlich ascitic plasma per tube. Each tube contained 3 ml phosphate buffer 0.1 M, pH 6.0, with incremental additions of BZAR and 40 μl of stock solution of substrate. The stock solution was 1 mM 4-methylumbelliferyl-*p*-guanidinobenzoate dissolved in dimethyl formamide. The inhibitor was incubated with the enzyme for 15 min prior to adding the substrate. The assay was carried out for 1 h at 37°C and the fluorescent product, 4-methylumbelliferone measured by fluorimetry.[1] The enzymic activity in the presence of BZAR is presented as a percentage of the activity of the enzyme in the absence of BZAR (Figure 1) plotted against the molarity of the BZAR in the assay. Inhibition was also demonstrated with Lineweaver-Burk kinetics with 3.5×10^{-6} M BZAR (Figure 2).

Location of cells in frozen sections containing active guanidinobenzoatase was

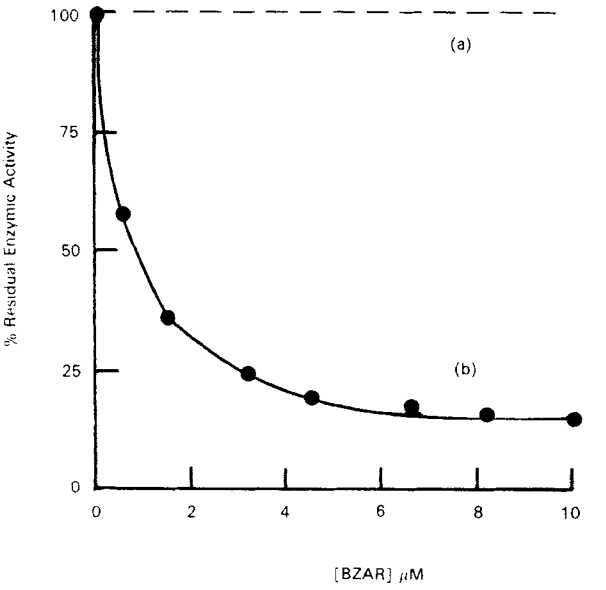


FIGURE 1 Inhibition of guanidinobenzoatase in solution by BZAR assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate. The broken line (a) represents the failure of BZAR to inhibit 2 μg of trypsin assayed with benzoylarginine-β-naphthylamide. The points, curve (b), represent the inhibition of guanidinobenzoatase by incremental additions of BZAR.

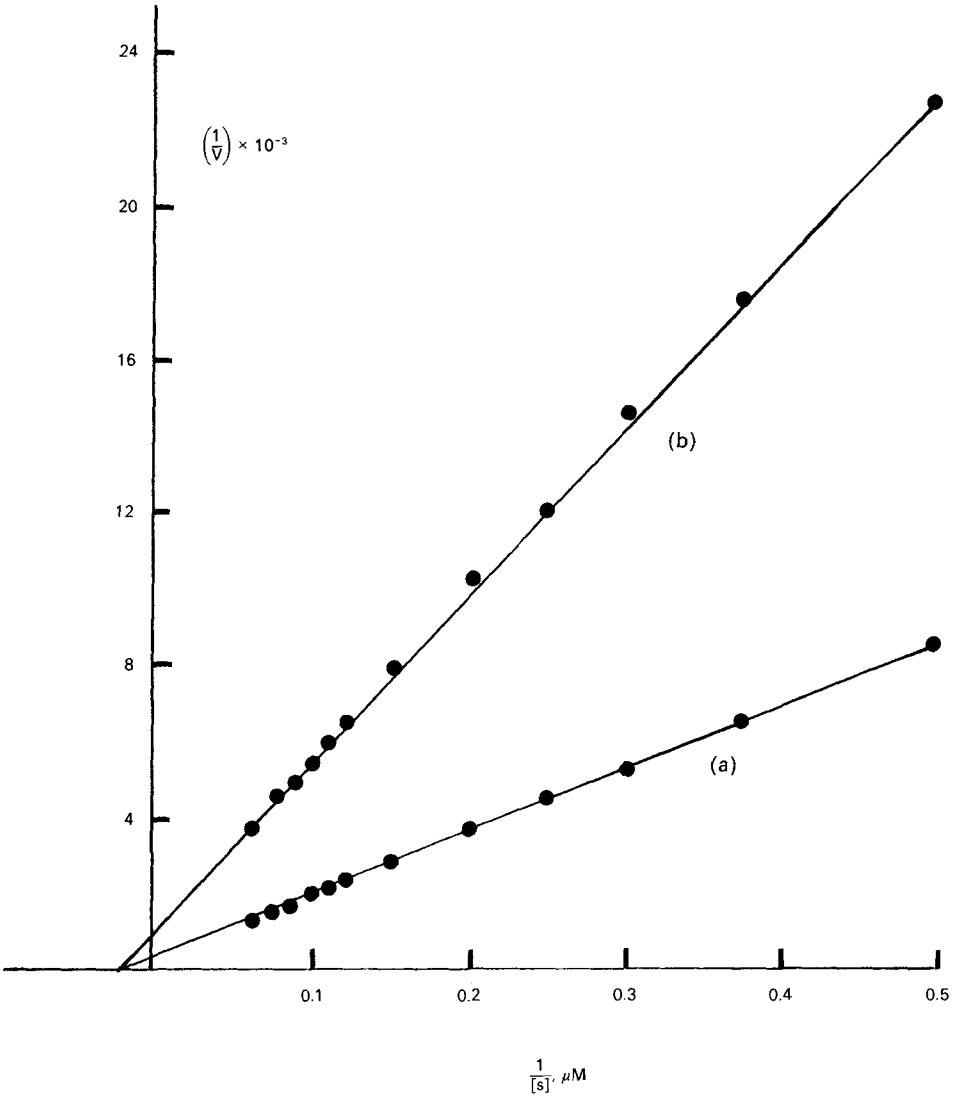


FIGURE 2 Lineweaver-Burk plots of the inhibition of guanidinobenzoatase by BZAR assayed with 4-methylumbelliferyl-*p*-guanidinobenzoate as substrate. (a) Enzyme alone, (b) Enzyme with BZAR (3.5×10^{-6} M.).

carried out as described previously[3] with the exception that the washing time in isotonic saline was reduced to 1 min. Briefly, the sections were placed in 10^{-3} M 9-aminoacridine in isotonic saline for 2 min, and washed for 1 min in fresh isotonic saline. Those cells possessing active enzyme[2] bound 9-aminoacridine and fluoresced yellow on a blue background. Sections from 20 different tumour bearing tissues were employed and the results are illustrated with tissue taken from patient J.K. who had a squamous cell carcinoma of the oral cavity. The effect of BZAR at 10^{-5} M in isotonic saline was studied as follows:

Fig. 3

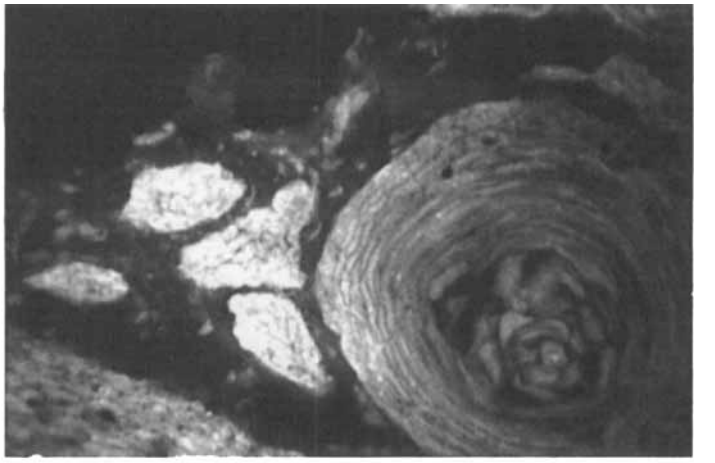
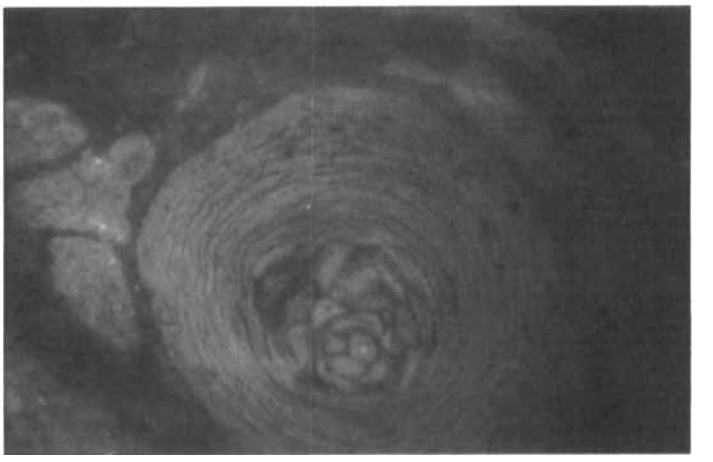


Fig. 4



FIGURES 3 AND 4 (see colour plates X and XI at the back of the issue). 9-Amino acridine staining of a section containing squamous cell carcinoma of the oral cavity. Figure 3 without BZAR pretreatment; Figure 4 with BZAR pretreatment. Magnification × 200, yellow filters used for photography and printing (in each case) to preserve the yellow fluorescence of 9-aminoacridine. Clearly the BZAR has inhibited the 9-aminoacridine binding in Figure 4.

One section had no BZAR treatment and this acted as the control (Figure 3). One section had a 2 h treatment with BZAR prior to staining with 9-aminoacridine (Figure 4). One section was treated with 9-aminoacridine first, prior to treatment with BZAR followed by a second staining with 9-aminoacridine.

RESULTS AND DISCUSSION

The inhibition of guanidinobenzoatase by BZAR in true solution is shown in Figure 1. The inhibition of guanidinobenzoatase by BZAR (3.5×10^{-6} M) assayed with

4-methylumbelliferyl-*p*-guanidinobenzoatase is presented in the form of a Lineweaver-Burk plot in Figure 2. This plot indicates that BZAR is a non-competitive inhibitor of guanidinobenzoatase with a K_i value of approximately 2×10^{-6} M. This K_i value is in agreement with the data presented in Figure 1, where 50% inhibition was achieved with 2 μM BZAR. It is obvious that BZAR is a good inhibitor of guanidinobenzoatase whilst this compound can be used as a fluorogenic substrate for trypsin and trypsin-like enzymes.[8] It was checked whether BZAR had any inhibitory action on trypsin assayed with N-benzoylarginine-β-naphthylamide and no inhibition could be observed at the concentrations used in Figure 1. It is of interest that the active site titrants designed to inhibit and quantitate trypsin[4,5] are both substrates for guanidinobenzoatase, whilst an inhibitor of guanidinobenzoatase (BZAR) is used in a fluorescent assay for trypsin-like enzymes.[8] This implied that BZAR can be used to selectively inhibit guanidinobenzoatase and would be expected to have no harmful effects on other essential trypsin-like enzymes in experiments involving whole animals. In view of the known activity of guanidinobenzoatase associated with tumour cells,[9] the inhibition of this enzyme by BZAR takes on a new significance.

It was observed that tumour cells containing active guanidinobenzoatase in frozen sections bound 9-aminoacridine and fluoresced yellow (Figure 3). The effect of BZAR on guanidinobenzoatase associated with tumour cells was examined in frozen sections of human tissues. It was observed that pretreatment of the frozen sections with 10^{-5} M BZAR completely blocked the binding of 9-aminoacridine to tumour cells (Figure 4). This indicated that BZAR occupied the same binding site on the guanidinobenzoatase as the 9-amino acridine, viz. the active centre. This binding of BZAR to the active centre was not reversed by competition with 9-aminoacridine and it is concluded that this was irreversible non-competitive binding of BZAR to the active centre. Conversely it was observed that when the section had been previously stained with 9-aminoacridine, BZAR was unable to displace the bound 9-aminoacridine. Non-competitive inhibition was also demonstrated in Figure 1 when the fluorogenic substrate was used to assay the enzyme. These findings support the claim of non-competitive binding of BZAR to the active centre of guanidinobenzoatase. The major requirement for binding to the active centre is a strongly protonated amino group[10] and this is provided by one of the guanidino groups in BZAR (Figure 5), simulating the arginine residue which is known to be the preferred cleavage point in synthetic peptides.[10]

We conclude that those tumour cells *in vivo* which possess active guanidinobenzoatase can be inhibited by BZAR. BZAR is a substrate for other trypsin-like

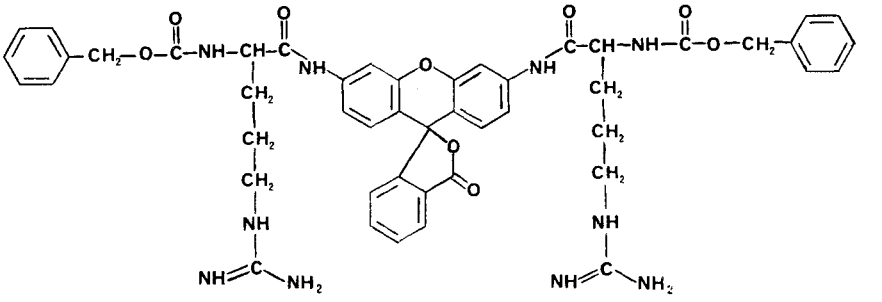


FIGURE 5 The structure of BZAR.

enzymes and this compound should not affect these trypsin-like enzymes *in vivo*. We know that guanidinobenzoatase is a tumour associated protease found in cells capable of migration and therefore believe that BZAR and its analogues may have a role in both tumour control and also the targeting of cytotoxic molecules to tumour cells.

Acknowledgements

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