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INHIBITION OF A TUMOUR PROTEASE WITH 3,4-DICHLOROISOCOUMARIN, PENTAMIDINE-ISETHIONATE AND GUANIDINO DERIVATIVES

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Guanidinobenzoatase (GB) is a cell surface proteolytic enzyme capable of degrading fibronectin, and is associated with tumour cells and cells capable of migration. The location of active GB in sections has been demonstrated with 9-aminoacridine (9-AA), a competitive inhibitor of GB. 3,4-Dichloroisocoumarin (3,4-DCI) and pentamidine isethionate (PI) are inhibitors of trypsin-like enzymes. It has now been demonstrated that 3,4-DCI, PI, and guanidino derivative compounds are significant inhibitors of GB, on the surfaces of lung squamous cell carcinoma cells in frozen sections and free GB in solution. Dexamethasone acetate (DMA) and medroxy-progesterone (MP) did not show any significant inhibition of GB activity. These molecules lack a reactive chloride or guanidino groups and are thought to react at the nuclear level, rather than directly on this cell surface protease. Kinetic studies have shown that 3,4-DCI, PI and guanidino derivatives are reversible competitive inhibitors of GB, as determined *in vitro* on the purified enzyme. The inhibition resulting with 3,4-DCI was a time-dependent process. It is suggested that these inhibitors interact with GB by binding to its active site, resulting in the formation of enzyme-inhibiter complexes (GB-I). The GB-I complexes can be dissociated with SDS treatment, resulting in the regain of GB activity.

KEY WORDS: Lung carcinoma, cell surface, protease, guanidinobenzoatase

INTRODUCTION

The invasion of cells of one tissue into the neighbouring tissues occurs during many physiological processes, in both normal and pathologic conditions. In all these processes the invading cells breach the barrier of extracellular matrix, which seems to be done by a common mechanism of releasing an array of proteolytic enzymes.[1]

Guanidinobenzoatase (GB) is a tumour associated protease,[2] now known to be similar to tissue plasminogen activator,[3] but not identical.[4] Previous studies have shown that the cell surface GB can be located by the yellow fluorescent, active site

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ABBREVIATIONS: Guanidinobenzoatase (GB); 4-Methylumbelliferyl-p-guanidinobenzoate (MUGB); 4-Methylumbelliferone (MU); 9-Aminoacridine (9-AA); 3,4-Dichloroisocoumarin (3,4-DCI); Pentamidine isethionate (PI); 2-Guanidinobenzimidazole (2-GB); 4-Acetamidophenyl guanidinobenzoate (4-AGB); Dexamethazone (DM); Medroxy-progesterone acetate (MPA); Sodium dodecyl sulphate (SDS); Plasminogen activators (PAs); Sulfaguanidine (SG).

directed competitive inhibitor 9-aminoacridine (9-AA). Cells which possess active GB bind 9-AA and fluoresce yellow under fluorescent microscopic conditions.[5] When GB is present in a latent form (enzyme-inhibitor complex), then 9-AA fails to bind to the GB and these cells do not exhibit yellow fluorescence. The enzyme-inhibitor complex can be dissociated with SDS treatment,[6] which results in the regain of cell surface GB ability to bind 9-AA and fluoresce yellow.

3,4-Dichloroisocoumarin (3,4-DCI) is a potent inhibitor of several serine proteases, but does not inhibit the thiol proteases.[7] Pentamidine isethionate (PI) is a non-peptide anti-parasitic effective drug, currently used for the treatment of *Pneumocystis carinii* pneumonia in AIDs patients,[8] and an effective inhibitor of cellular tumour necrosis factor[9] (released from lipo-polsaccharide-stimulated rat alveolar macrophages), at a concentration of 10^{-8} M. PI is also a known inhibitor of trypsin-like enzymes, and prevents the early differentiation of cultured cells by preventing cell adherence to plastic and glass surfaces.[10] Dexamethasone (DM) and medroxy-progestrone acetate (MPA) are steroid-like drugs which have been used in tumour cell studies[11,12] to suppress metastatic potential.

In the present study, we have explored the effect of 3,4-DCI, PI, and guanidino derivatives such as 2-guanidino-benzimidazole (2-GB), 4-acetamidophenyl guanidinobenzoate (4-AGB), sulfaguanidine (SG), (DM) and (MPA) on GB, associated with lung squamous cell carcinoma by fluorescent microscopic studies of membrane-bound GB and kinetic studies of free GB in solution.

MATERIALS AND METHODS

Fresh lung tumours and frozen blocks of lung tumours, which have been defined as squamous cell carcinoma, were kindly provided by the Department of Pathology, Wythenshawe Hospital Manchester. Cryo-sections were cut in the Histology Department, University of Manchester.

4-Methylumbelliferyl-p-guanidiobenzoate (MUGB), 9-amino-acridine (9-AA), 3,4-dichloroisocoumarin (3,4-DCI), pentamidine isethionate (PI), 2-guanidinobenzimidazole (2-GB), sulfaguanidine (SG), dexamethasone (DM), and medroxyprogesterone acetate (MPA) were purchased from Sigma Chemical Co. Ltd; St. Louis, Mo, USA. 4-Acetamidophenyl guanidinobenzoate (4-AGB) was purchased from Aldrich Chemicals.

Direct 9-AA Staining

Direct 9-AA staining was carried out[2] by placing the slides in 300 ml isotonic saline, containing 10^{-3} M 9-AA for 2 min, followed by washing the excess stain from the slides in fresh isotonic saline for 20 s. Protected and formaldehyde treated frozen sections, were prepared as previously described,[3] to provide cells lacking cytoplasmic inhibitors, but with active GB attached to the cell surfaces.

Assay of GB in Solution

GB (20 μg/ml) was assayed with the fluorogenic substrate, MUGB (2 x 10^{-5} M final concentration), in phosphate buffer, pH 6.1 at 37°C. The fluorescent product, methylumbelliferone (MU) was measured by an Aminco-Bowman fluorescence spectrophotometer.[2] Cleavage of the substrate was monitored at an excitation wavelength 323 nm and emission wavelength 446 nm.

Inhibition of GB

GB was isolated and purified from lung squamous cell carcinoma by affinity chromatography,[5] with slight modification. Hypertonic saline was used to displace the GB bound to the agmatine-sepharose affinity column.[14] Inhibition experiments were carried out by pre-incubating freeze dried GB (20 μg/ml) with variable amounts of inhibitors (0.5 x 10^{-5} to 1 x 10^{-2} M) for 10 min at 37°C prior to adding the MUGB substrate (2 x 10^{-5} M final concentration). Dixon plots were used to define the type of inhibition, by plotting 1/v versus the concentration of inhibitors, with two different substrate concentrations (2 x 10^{-5} and 2 x 10^{-4} M).

Fluorescence Microscopy and Photography

Sections stained with 9-AA were examined in the Leitz fluorescence microscope with cube G (Leitz catalogue No 513602). The microscope was fitted with an automatic camera and Kodak ASA 400 colour film was used to record the data. Under these conditions cells with active GB exhibited yellow surface fluorescence and the cells lacking active GB appear as green-yellow.

RESULTS AND DISCUSSION

Protected and formaldehyde treated cryo-sections, which lack local inhibitors but have active GB,[3] were directly stained with 9-AA. The cells possessing active GB bound 9-AA and fluoresce yellow (Figure 1). When sections were treated with 4×10^{-5} to 4.5×10^{-4} M 3,4-DCI, PI, 2-GB, 4-AGB and SG for 1 hour, followed by 9-AA staining, the surfaces of the tumour cells failed to fluoresce yellow (Figure 2). After treating these sections with SDS (10^{-2} M) for 10 minutes, and extensively washing in isotonic saline, the cell surface GB regained its ability to bind 9-AA and fluoresced yellow (Figure 3).

These results suggest that 3,4-DCI, PI, 2-GB and 4-AGB interact with cell surface GB by binding to its active site, leading to the formation of enzyme-inhibitor complexes (GB-I) on the cell surfaces, which then fails to bind 9-AA. After SDS treatment the GB-I complexes were dissociated,[6] and 9-AA regained its ability to bind to the active sites of GB and enabled the cell surfaces to fluoresce yellow (Figure 3). Secondly, when these sections (having GB-I complexes) were put in a large tank of 9-AA for 20 minutes, the inhibitors were displaced by 9-AA, and the cell surfaces fluoresced yellow (data similar to Figure 3), suggesting that 3,4-DCI, PI, 2-GB, 4-AGB and SG are competitive inhibitors of GB. When the protected sections of lung carcinoma were

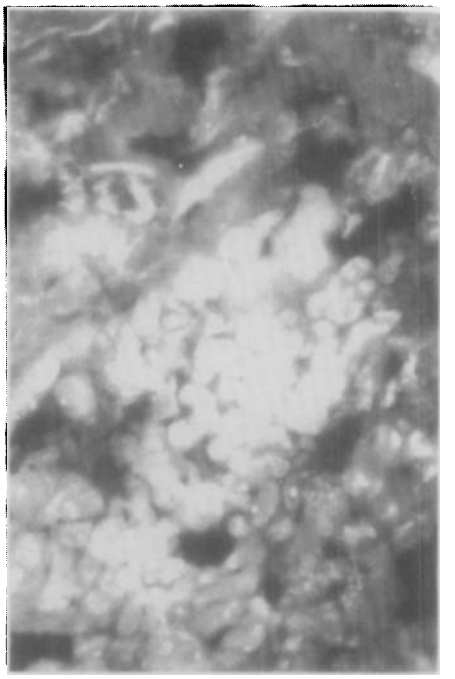
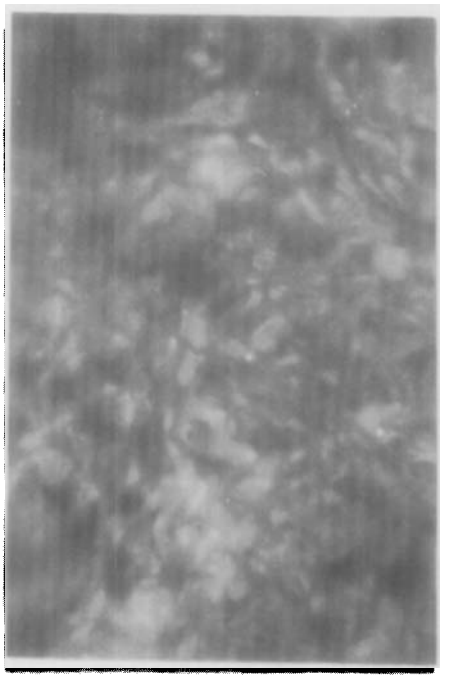


FIGURE 1 Protected cryo-section of lung squamous cell carcinoma tissue directly stained with 9-AA. The surfaces of the tumour cells which possess active GB, bind 9-AA and fluoresced yellow. The surrounding cells lack this cell surface GB and hence lack fluorescence. Magnification × 250. See Color Plate IV

FIGURE 2 Protected cryo-section of lung squamous cell carcinoma, after treatment with 3,4-dichloroisocoumarin (4.5×10^{-5} M) for 1 h, followed by 9-AA staining. The cell surfaces of the lung carcinoma cells fail to fluoresced yellow, due to the prior formation of enzyme-inhibitor complex (GB-I) on the surfaces of cells. 9-AA did not bind to the GB on the cell surfaces, and the section appears green yellow. Magnification × 250. See Color Plate V



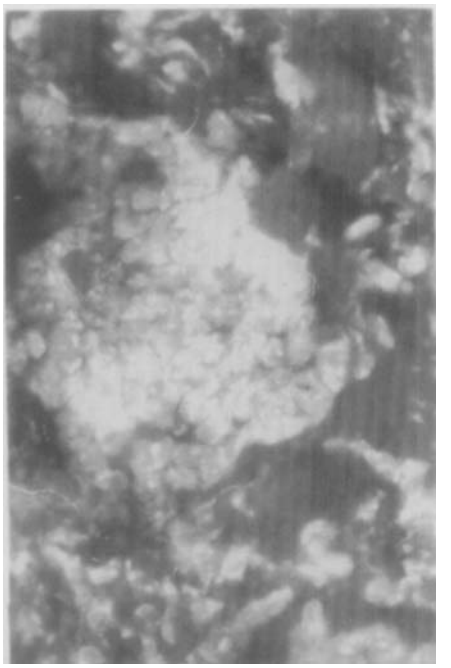
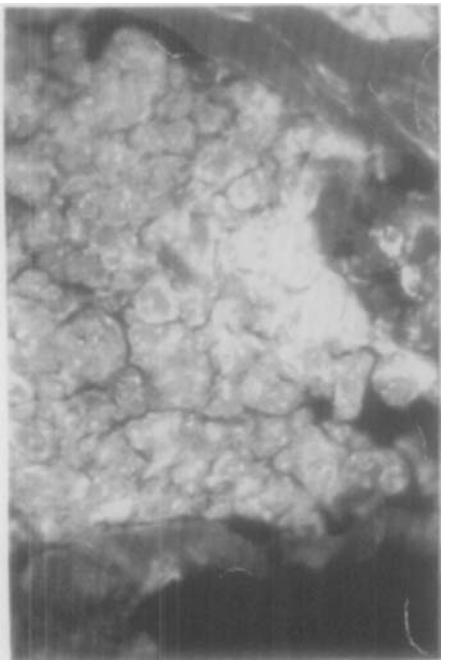


FIGURE 3 Section shown in Figure 2, after displacement of 3,4-dichloroisocoumarin with SDS (10^{-2} M), followed by 9-AA staining. The GB on the lung squamous cell carcinoma in this section, after displacement of inhibitor from the active site with SDS, regained its ability to bind 9-AA, and the cell surfaces now fluoresced yellow. Magnification × 250. See Color Plate VI

FIGURE 4 Protected section of lung squamous cell carcinoma after dexamethasone treatment (10^{-2} M), for 1 h, followed by 9-AA staining. The tumour cell surfaces in this section fail to recognise and bind the dexamethasone, and after binding 9-AA, the cell surfaces fluoresced yellow. Magnification × 250. See Color Plate VII



treated with (4.5×10^{-4} to 1×10^{-2} M) SG, DM and MPA for 1 hour, the GB on the cell surfaces remained active and the sections fluoresced yellow after 9-AA staining (Figure 4).

Kinetic studies have shown that soluble GB, extracted from lung tumours was capable of cleavage of MUGB, an active site titrant for trypsin-like enzymes,[13] with a consequent release of the fluorescent product, 4-methylumbelliferone (MU). The addition of 3,4-DCI, PI, 2-GB, 4-AGB and SG to a final concentration of 4×10^{-5} to 4.5×10^{-4} M caused a marked reduction in MU production (Figures 5-7). When the inhibition of GB was presented in the form of Dixon plots (Figure 8) with two different substrate concentrations (2×10^{-5} M and 2×10^{-4} M), it was confirmed that 3,4-DCI, PI, 2-GB, 4-AGB and SG were all competitive inhibitors of GB, with a calculated apparent K_i value of 2.46×10^{-8}, 2.39×10^{-7} M, 5.17×10^{-7} M, 6.24×10^{-7} M and 1.13×10^{-6} respectively. The inhibition noted with 3,4-DCI was a time-dependent process, and after 2 hours incubation complete inhibition was observed. These findings are in agreement with Harper *et al*'s[7] observation on the rate of inactivation of different serine proteases by 3,4-DCI.

DM and MPA up to 10^{-2} M, did not cause any significance inhibition (Table 1, Figure 6). The results obtained with DM and MPA were not in agreement with the previous studies on plasminogen activators (PAs) in culture experiments. Markus *et al*[11] demonstrated that DM, a glucocorticoid effectively inhibits PAs secretion in short-term organ culture of human adenocarcinoma of breast. The angiostatic steroid MPA (10^{-6} M), has been shown to inhibit vascularisation, collagenolysis and PAs levels in the culture medium by 39%. MPA decreased PAs activity and this was found to be mediated by a significance increase in the PAs inhibitor type-1 production.[12]

From the above observations it is concluded that in cultured cells DM and MPA inhibit the production of PAs at the nuclear level and fail to show a significance effect on membrane-bound GB in sections and free GB in solution. On the other

TABLE 1
Inhibition of GB with different inhibitors, assayed with substrate MUGB at a final concentration of (2×10^{-5} M).

Inhibitors	concentration (M)	residual activity (%)	K_i (M)
None	–	100	–
3,4-DCI	4.0×10^{-5}	00	$2.46\ 10^{-8}$
PI	4.5×10^{-4}	08	2.39×10^{-7}
2-GB	4.5×10^{-4}	18	5.17×10^{-7}
4-AGB	4.5×10^{-4}	22	6.24×10^{-7}
SG	4.5×10^{-4}	34	1.13×10^{-6}
DM	1.0×10^{-2}	81	N.D.
MPA	1.0×10^{-2}	99	N.D.

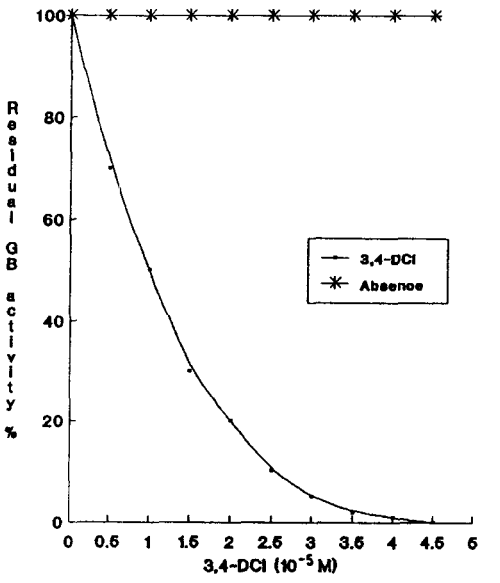


FIGURE 5 Effect of 3,4-dichloroisocoumarin (0-4.5 × 10^{-5} M) on GB activity, assayed with MUGB at 37°C. The enzyme and inhibitor was incubated for 2 h to obtain complete inhibition. The reduction of MU was a time-dependent process. 100% activity of 20 μg/ml freeze dried GB was equivalent to 2 nmol of MU produced per minute.

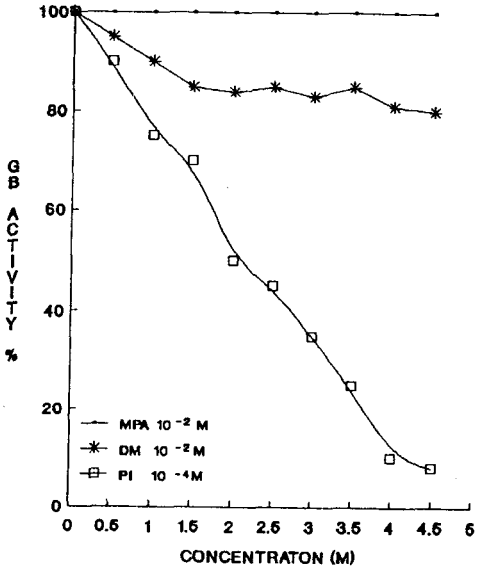


FIGURE 6 Effect of pentamidine isethionate (0-4.5 × 10^{-4} M), dexamethasone (0-4.5 × 10^{-2} M) and medroxy-progestron (0-4.5 × 10^{-2} M) on GB in solution assayed with MUGB. The production of MU was markedly reduced (92%), with an increase in pentamidine isethionate concentration, but dexamethasone and medroxy-progestron did not show a significant inhibition.

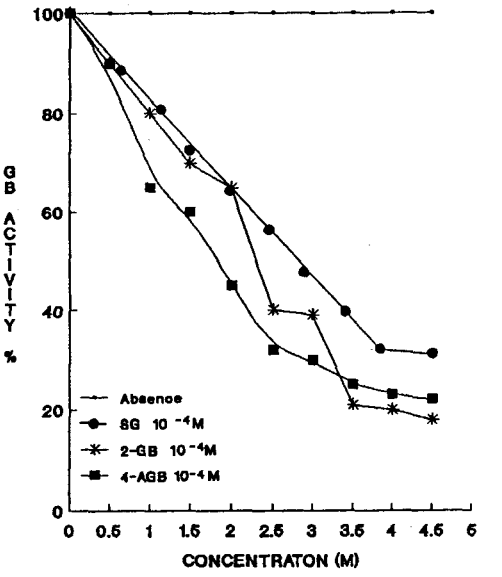


FIGURE 7 The inhibition of GB activity with 2-guanidino-benzimidazole, 4-acetamidophenyl guanidinobenzoate and sulfaguanidine, assayed with MUGB at 37°C. GB activity was significantly reduced with the addition of inhibitors to a final concentration of 4.5×10^{-4} M.

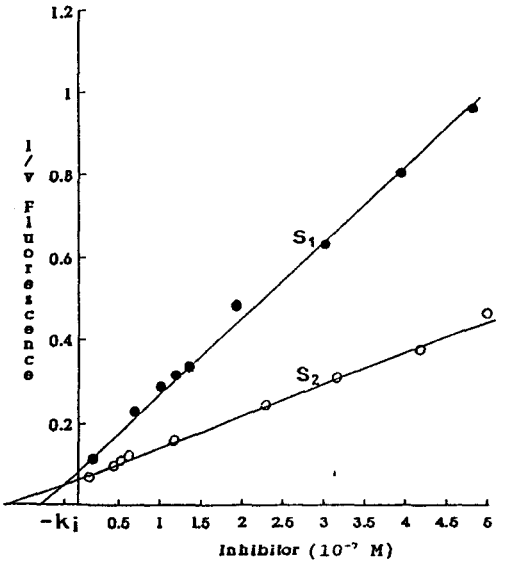


FIGURE 8 Dixon plot of 1/v versus inhibitor, 3,4-dichloro-isocoumarin (0-4.5×10^{-5} M) for the inhibition GB using the substrate MUGB at a final concentration of 2×10^{-5} M (S1) and 2×10^{-4} M (S2). The final concentration of GB was 20 μg/ml. The common intercept revealed a competitive type of inhibition with a calculated apparent K_i of 2.46×10^{-8} M.

hand 3,4-DCI, PI, 2-GB, 4-AGB and SG significantly reduced cell bound GB activity (data similar to Figure 2). This effect can be reversed by putting the inhibitor treated sections in a large tank of isotonic saline containing 10^{-3} M 9-AA, suggesting that these inhibitors are competitive inhibitors of cell surface GB. Furthermore, 3,4-DCI, PI, 2-GB, 4-AGB and SG bind to the active sites of GB, resulting in the formation of enzyme-inhibitor complexes (GB-I) which can be dissociated with SDS.[6] This inhibition of cell surface GB by these inhibitors was further supported by the kinetic data (Figure 6, 7), in which these inhibitors have been shown to cause significant inhibition of GB activity in solution.

Acknowledgements

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References

1. Moscatelli, D. and Rifkin D.B. (1988) *Biochim. Biophys. Acta.* **948**, 67–85.
2. Steven, F.S., Al-Ahamad and Griffin, M.M. (1983) *Eur. J. Biochem.* **130**, 335–339.
3. Steven, F.S., Griffin, M.M. and Williams, L.A. (1991) *Anticancer Res.*, **11**, 641–47.
4. Delpont, C.P., Descomps, R. and Aberger, P. *Cancer Res.* **52**, 3622–3628.
5. Steven, F.S., Griffin, M.M. and Al-Ahmad (1985) *Eur. J. Biochem.*, **149**, 35–40.
6. Steven, F.S., Griffin, M.M. and Blakey, D.C. (1992) *J. Oncology* – 59–61.
7. Harper, J.W., Hemmi, K. and Powers J.C. (1985) *Biochem.*, **24**, 1831–1841.
8. Connor, T.H. and Trizna, Z. (1992). *Toxicol. Lett.*, **63**, 69–74.
9. Corsini, E. Craig, W.A. and Rosenthal, G.J. (1992) *Int. J. Immuno. Pharmacol.*, **14**, 121–30.
10. Klemes, Y. (1984) *Differentiation*, **27**, 141–145.
11. Markus, G., Camiolo, M.S. and Kohga (1983) *Cancer Res.*, **43**, 5517–5525.
12. Blei, F., Wilson, E.L., Minatti, P. and Rifkin, D.B. (1993) *J. Cell Physiol.*, **155**, 568–78.
13. Coleman, P.L. Lathman, H.G. and Shaw, E.N. (1976) *Meth. Enzymol.*, **45**, 12–26.
14. Anees, M. and Steven, F.S. (1994) *J. Enz. Inhib.* (in press).