J. Enzyme Inhibition, 1992, Vol. 5, pp. 299–315
Reprints available directly from the publisher
Photocopying permitted by license only

© 1992 Harwood Academic Publishers GmbH
Printed in the United Kingdom

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

THE ROLE OF FIBRIN FIBRILS IN THE DISSOCIATION OF A CELL SURFACE PROTEASE-INHIBITOR COMPLEX AND EVIDENCE FOR THE RECAPTURE OF THE INHIBITOR PROTEIN

F.S. STEVEN†‡ and D.C. BLAKEY§

‡Deparment of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester M13 9PT, U.K. and §ICI Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, SK10 4TG, U.K.

(*Received 10 July 1991*)

Colonic epithelial cells possess a cell surface protease referred to as guanidinobenzoatase (GB). Active GB can be located by the fluorescent active site directed competitive inhibitor 9-amino acridine (9AA) followed by fluorescence microscopy. The cell surface GB can be transferred to fibrin fibrils, which have a higher affinity for GB than the cell surface. The cytoplasm of colonic epithelial cells contains a protein which inhibits membrane bound GB, forming a latent form of GB or GB-inhibitor complex. This complex can also be dislodged from the epithelial cell surface due to the high affinity of fibrin for GB, with the consequent dissociation of the enzyme-inhibitor complex and solubilisation of the inhibitor. This use of fibrin has led to the demonstration of the transfer of a selective inhibitor protein from one cell surface (the donor) to a second cell surface (the target).

KEY WORDS: Guanidinobenzoatase, fibrin, enzyme-inhibitor complex, recapture of inhibitor.

INTRODUCTION

Plasminogen activator (PA) occurs as tissue plasminogen activator and urokinase;[1] these two forms of PA are present as soluble proteins and as membrane bound enzymes, the latter are thought to be attached to cell surface receptor proteins.[2] The most studied function of PA is the conversion of plasminogen into plasmin, to initiate the proteolytic degradation of fibrin clots.[3] This activation of plasminogen by PA is greatly facilitated by the binding of PA to fibrin fibrils and in this reaction the fibrin has been considered to be an essential cofactor in the activation process.[4] PA is thought to play a significant role in cell migration, in which the enzyme is generally considered to be linked through a receptor protein[5] located on the cell surface.[6] In the case of tumour cell migration there is considerable debate whether t-PA or u-PA is the form of PA primarily associated with transformed cells and tumour cells,[7,8] although it is agreed that most transformed cells and tumour cells possess increased levels of PA activity.

Earlier studies from this laboratory[9] described a tumour associated protease, referred to as guanidinobenzoatase (GB) on account of its unusual substrate specificity in cleaving guanidinobenzoates, which inhibited other seryl proteases. GB in solution was competitively inhibited[10] by 9-aminoacridine (9AA). It was realised that the affinity of 9AA for GB could be used to locate cells possessing active GB, since

†Correspondence.

RIGHTSLINK

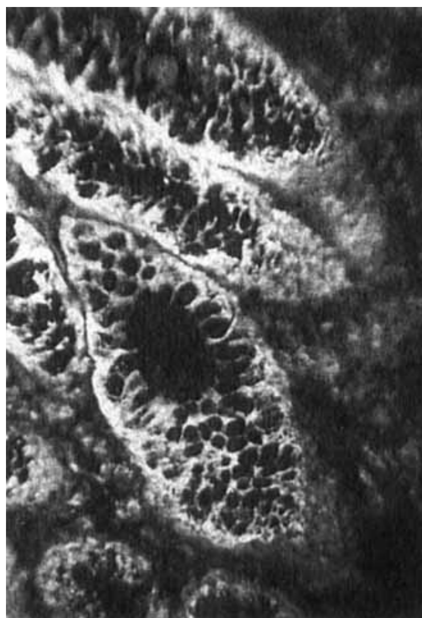


FIGURE 1 Direct 9AA staining of frozen section. The colonic epithelial cells of the crypts possess active GB (yellow), some lymphocytes are also fluorescent (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

the 9AA could be used to generate a fluorescent signal under appropriate microscopic conditions.[10,11] The inhibitor of PA (referred to as PA1-1) was shown to react with cell surface GB resulting in the inhibition[12] of GB when exposed to 9AA, whilst a polyclonal antibody directed against t-PA also inhibited the binding of 9AA to GB on cell surfaces.[13] The peptide inhibitor of t-PA, Dans-GGACK[14] was also shown to inhibit the binding of 9AA to these cells[13] leading to the conclusion that GB and t-PA were functionally very similar proteins.[13] This conclusion has recently been opposed by studies on cells in culture,[15] in which the guanidinobenzoatase activity measured by the cleavage of *p*-nitrophenyl-*p*-guanidinobenzoate was not inhibited by monoclonal antibodies to PA. In the present report we include studies with guanidinobenzoates and compare our results with those of Perry and Scott.[15] For the present time we think it advisable to refer to the cell surface protease which binds 9AA as GB. The name of the enzyme is less important than the exchange reactions with its inhibitor, which we are about to describe.

Active GB can be located on cell surfaces by the binding of the fluorescent probe 9AA followed by examination under appropriate microscopic conditions.[10,11] Cells which possess GB also possess cytoplasmic inhibitors which recognise the cell surface GB and form enzyme-inhibitor complexes which can be dissociated by treatment with formaldehyde resulting in the regain[16] of the cell surface GB's ability to bind 9AA. These inhibitor proteins may play a role in regulating the activity of cell surface GB *in vivo*[16] and their ability to recognise the cell surface GB is of considerable interest in view of the PA-like activity[13] of GB and its possible role in cell migration and metastasis.[5,12]

RIGHTSLINK

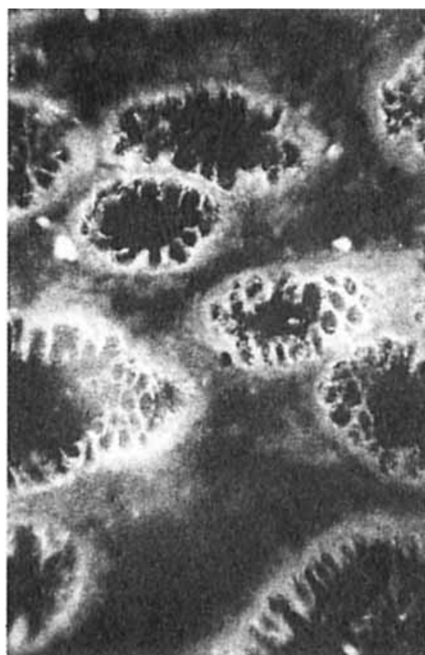


FIGURE 2 Prior exposure of section to 10 μl isotonic saline followed by 9AA staining. The GB on the surfaces of epithelial cells and lymphocytes no longer binds 9AA and these cells appear pale green. This inhibition can be reversed (see Figure 3). Prolonged exposure times cause the blurring of the final photograph (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

The purpose of the present study was to demonstrate the role of fibrin fibrils in the dissociation of the latent GB-inhibitor complex from the cell surface with the subsequent capture of GB on fibrin and release of active inhibitor molecules. the released inhibitor molecules were then used to interact with GB on the surface of cells from which the original cytoplasmic inhibitors had previously been removed.[17] We were thus able to demonstrate (a) the formation of an enzyme-inhibitor complex on the cell surface, (b) the role of fibrin in dissociating the enzyme-inhibitor complex from the cell surface, (c) the dissociation of the enzyme inhibitor complex into free inhibitor and fibrin bound enzyme, and (d) the transfer of this inhibitor and its recapture by GB on *protected*[17] cells. These interactions on the cell surface have been followed by using the fluorescent probe 9AA as a reversible competitive inhibitor of the cell surface enzyme and following the course of these transfers by fluorescent microscopy of appropriate frozen sections of colonic epithelial cells, which possess both GB and an inhibitor of GB. The normal human colon contains a variety of cells possessing GB, for example the epithelial cells of the crypts and villi which are easily recognised by their morphology, and the surrounding lymphocytes. In this study we present evidence obtained from the crypts located adjacent to the muscle layer since these structures are easily recognised by those unqualified in histology.

RIGHTSLINK

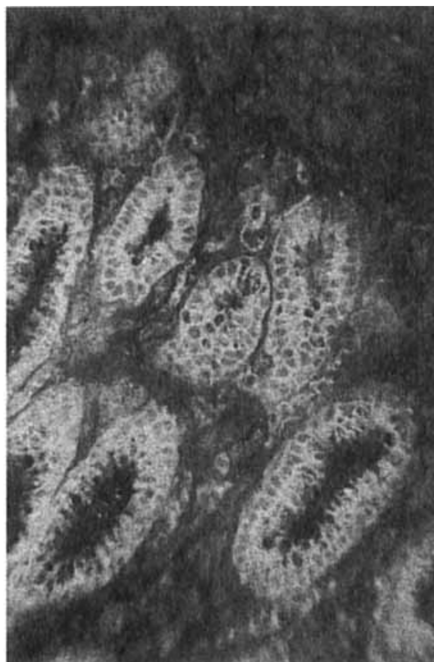


FIGURE 3 Displacement of inhibitor with formaldehyde followed by 9AA staining. Formaldehyde treatment of the section used in Figure 2 resulted in a regain of the epithelial cells ability to bind 9AA, due to the displacement of the inhibitor from the E-1 complex. The cells now bind 9AA and fluoresce yellow, similar to Figure 1 (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

MATERIALS AND METHODS

Normal human colonic tissue obtained at surgery was provided by a local hospital and cut as multiple frozen sections (4 μm) at ICI, Alderley Park. These sections were then transferred in dry ice to the University of Manchester for analysis in the Department of Biochemistry and Molecular Biology. In this study we are only concerned with the cell surface proteases of normal colonic epithelial cells in order to simplify the presentation of our results.

All the reagents used in this study were purchased from Sigma Chemical Company, St. Louis, Mo. USA. Stock solutions used were as follows: 9AA, 10^{-3} M in isotonic saline; formaldehyde 4% in isotonic saline, pH 7.2; thrombin from bovine plasma 10.5 NIH units/ml phosphate buffered saline; fibrinogen (7 mg/ml 0.1 M Tris HCl, pH 7.2). PD-10 columns of G-25M Sephadex were obtained from Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

Direct staining with 9AA

The frozen sections were placed in a tank (200 ml) of isotonic saline containing 9AA (10^{-3} M) for 2 min, drained of excess reagent and then washed free of unbound 9AA by placing in a tank of 200 ml fresh isotonic saline for 1 min.[11]

RIGHTSLINK

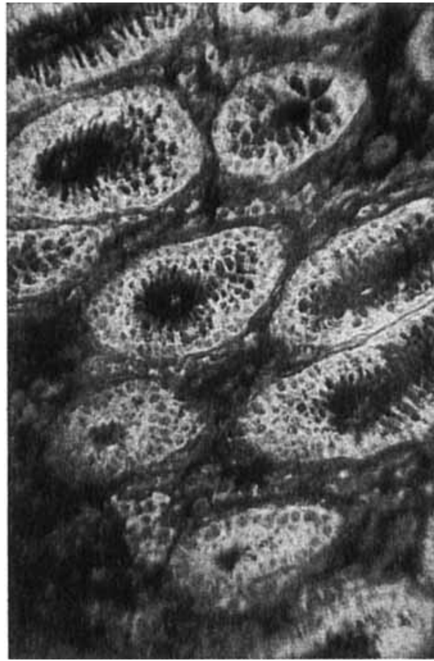


FIGURE 4 *Protected* section stained with 9AA. In this section the local cytoplasmic inhibitors have been washed out in the presence of 9AA. The cells possess active GB but lack the possibility of internal inhibitors reacting with the cell surface GB in subsequent experiments (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

Binding of cytoplasmic protein inhibitors to cell surface GB

The fresh frozen sections were covered with a thin film of isotonic saline (10 μl/section)[16] and placed in a wetbox for 1 h prior to 9AA staining as described above.

Protected sections

The techniques for the preparation of *protected* sections has already been described in detail.[17] This technique provides frozen sections containing membrane bound GB but lacking extractable cytoplasmic inhibitors of GB. The same procedure was used in this study.

Formaldehyde treatment of sections

Sections were placed in 4% formaldehyde pH 7.2 in isotonic saline for 18 h,[16] followed by washing and 9AA staining as described above.

Transfer of cell surface GB to fibrin fibrils from protected *sections*

Protected sections were placed under a layer of fibrinogen (100 μl) and 5 μl thrombin added to each layer prior to placing in a wetbox for 1–2 h. The fibrin clots were carefully removed from the surface of the sections prior to 9AA staining of these

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

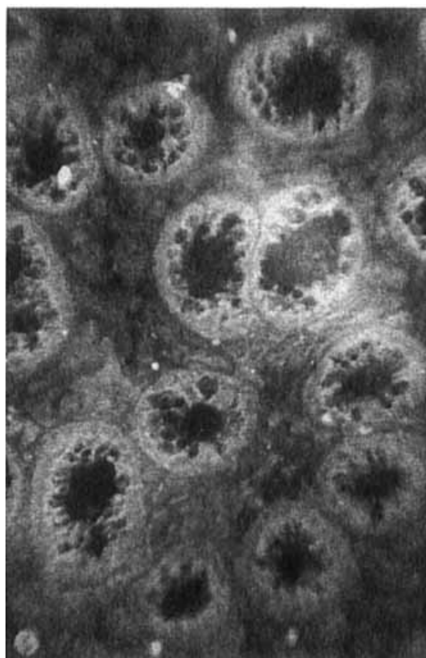


FIGURE 5 *Protected* sections exposed to fibrin prior to 9AA staining. The cell surface GB associated with *protected* sections (Figure 4) is not evident after exposure to fibrin which has a high affinity for plasminogen activator. The fibrin removes GB from the cell surface (see Figure 6) (see colour plate at rear).

fibrin-treated sections. Further treatment with formaldehyde[16] on these sections was carried out to release inhibitor and reactivate any latent GB.

Transfer of cell surface GB to fibrin fibrils from latent GB

This procedure was designed to dissociate the enzyme-inhibitor complex on cell surfaces by the presence of fibrin, with the subsequent collection of the soluble inhibitor fraction. It was necessary to saturate the cell surface enzyme with inhibitor molecues[16] extracted from the cytoplasm of the normal colonic epithelial cells of the section. This was achieved by placing 10 μl of isotonic saline on the surface of the sections (see above for binding of inhibitors to cell surface GB). At the ened of this preparative stage, sections were stained with 9AA and demonstrated to be negative, (i.e. good evidence for enzyme-inhibited complex formation which could be confirmed by sacrificing one of these sections for formaldehyde treatment). These frozen sections containing cell bound latent enzyme were then washed for 1 h in a tank of fresh isotonic saline (200 ml) to remove all soluble components and leave the membrane bound enzyme-inhibitor complex on the cell surfaces. The sections were then overlaid with fibrin (see above) and the fibrin clots collected after 1–2 h contact with the sections. The washed sections were stained with 9AA before and after exposure to formaldehyde[16] to check the total removal of cell surface GB by the fibrin. The fibrin clots were combined and centrifuged to provide a soluble fraction 300–400 μl which contained the potential inhibitor fraction (I). Fraction (I) was applied to the

RIGHTSLINK

FIGURE 6 Formaldehyde treatment of section previously exposed to fibrin. The cells lack active GB even after a treatment which is known to reactivate latent GB or GB-I complex (see Figures 2 and 3). The data indicate that fibrin removes GB from the cell surface rather than causing GB-I formation (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

surface of *protected* sections of colonic tissue and the possible presence of an inhibitor of GB demonstrated by subsequent 9AA staining before and after formaldehyde treatment of these sections.

Preparation of Rh-I

Six frozen sections of normal colon were taken which were known to possess typical crypts and villi and 10 μl of isotonic saline added to the surface of each section. After 1 h in a wetbox, the excess fluid was washed from the slides and the slides immersed in a tank (200 ml) of fresh isotonic saline for 1 h to remove all the soluble cytoplasmic components from the slide. As a result epithelial cells were obtained with membrane bound GB-inhibitor complex free of excess cytoplasmic inhibitor. These cells would not bind 9AA due to the presence of latent enzyme, (or enzyme inhibitor complex) rather than active GB on their surfaces. Next, a fibrin gel was formed on the surface (see above), the fibrin transferred to a centrifuge tube after 1 h and the supernatant soluble fraction collected. This soluble fraction (300 μl) was reacted with rhodamine isothiocyanate (10^{-6}M) at pH 8.0 for 1 h, then passed over a PD-10 column. The rhodamine labelled protein fraction (Rh-I) was freeze dried, dissolved in 100 μl water and 20 μl of this solution applied to the surface of a series of *protected* sections of normal colon. After 2 h the excess fluid was washed from the surface of the slides and the slides washed for 30 min in isotonic saline before examination for rhodamine

RIGHTSLINK

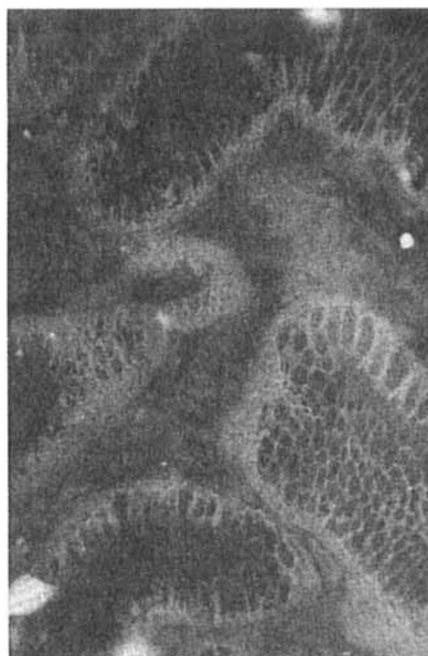


FIGURE 7 Transfer of membrane bound inhibitor via fibrin to *protected* sections. We first prepared sections in which the membrane bound GB was totally inhibited (similar to Figure 2), and coated these sections with fibrin. The potential inhibitor isolated from the fibrin was then used to challenge the GB on *protected* sections (similar to Figure 4) prior to staining with 9AA. Clearly the GB failed to bind 9AA, indicating inhibition of the cell surface GB in the target sections (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

fluorescence under the Zeiss microscope fitted with a cube designed specifically for fluorescent location of rhodamine (see Figures 9 and 10). Under these conditions rhodamine labelled proteins, (in our case Rh-I), appear red on a dark background.

Competition for cell surface GB between MUGB and 9AA

The 4-methylumbelliferyl-p-guanidinobenzoate (MUGB) was dissolved in dimethylformamide to give a stock solution of 1.34×10^{-3} M. Suitable dilutions of this stock in isotonic saline were prepared for competitive experiments with and without 9AA as follows: (A) 6.5×10^{-5} M MUGB in isotonic saline, (B) 6.5×10^{-5} M MUGB and 10^{-3} M 9AA in isotonic saline, (C) 10^{-3} M 9AA in isotonic saline.

A series of *protected* sections of colonic tissue were used in which the GB acted as targets for these active site directed agents. When the target GB was exposed to 100 μl MUGB in solution (A) for 3 min, followed by 100 μl solution (B) for 2 min and 1 min wash in saline, the effect of MUGB on the subsequent competition with 9AA for the active centre of cell bound GB could be monitored (Figure 11). The possibility that prolonged exposure (10 min) to 9AA (200 μl 10^{-3} M) would replace MUGB (in Figure 11) was checked, in which case the cell bound GB would now bind 9AA (Figure 12).

RIGHTSLINK

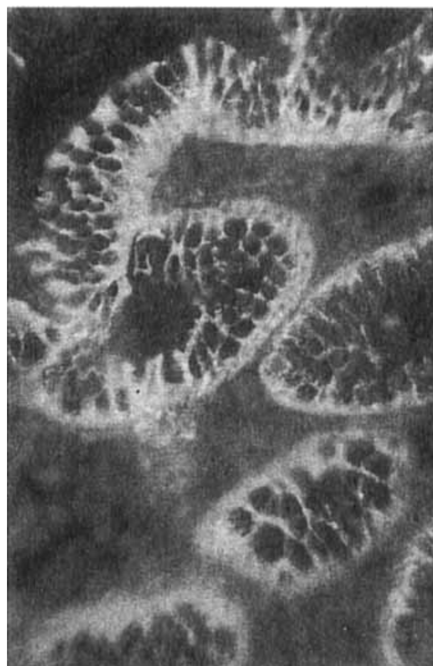


FIGURE 8 Formaldehyde displacement of transferred inhibitor. Sections in which the cell surface GB was inhibited by the fibrin-transferrred fraction (Figure 7) regained GB activity after formaldehyde displacement of inhibitor (see colour plate at rear).

Competition for cell surface GB between MUGB and the cytoplasmic inhibitor of GB

The protein inhibitors of GB used in this test series of experiments were obtained from the cytoplasm of the cells in frozen sections of normal colonic tissue. 10 μl isotonic saline containing 5.3×10^{-6} M MUGB was placed on the surface of these sections. After 1 h in a wetbox the test sections were washed with isotonic saline 200 μl and then placed in a tank (200 ml) of 9AA (10^{-3} M) dissolved in isotonic saline for 2 min followed by 1 min washing in isotonic saline (200 ml). As controls, corresponding sections were used in which 10 μl of isotonic saline lacking MUGB had been placed on the surface of the frozen sections. If the MUGB had offered protection to the GB on the surface of the epithelial cells in the presence of the extracted protein inhibitor of GB, the test system would bind 9AA (Figure 13) whilst the controls would not do so (similar to Figure 2).

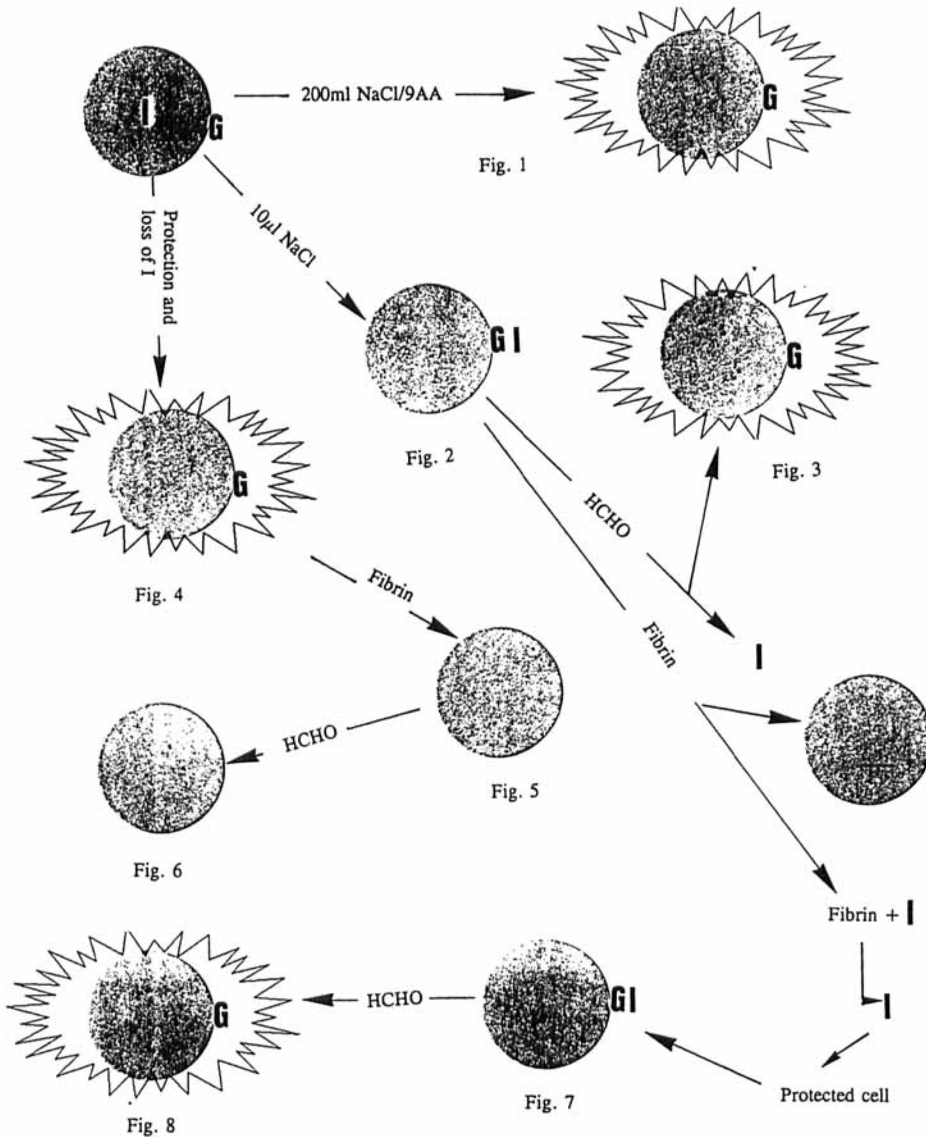
Microscopy

The location of GB on colonic epethial cell surfaces with 9AA was followed by the yellow fluorescence of stacked 9AA on cells with active GB.[11] For this study the Leitz Diaplan fluorescence microscope was used with filter cube [G] (Leitz catalogue no. 513602). Under these conditions cells with active GB fluoresced yellow, whilst those with latent GB or lacking GB appeared pale blue green. The Zeiss microscope used was fitted with a cube designed for rhodamine fluorescence for the location of colonic

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.



SCHEME I The darkened circle represents the cell in a frozen section. I represents the cytoplasmic inhibitor of the cell surface protease (abbreviated to G). GI represents latent enzyme or enzyme-inhibitor complex and HCHO represents formaldehyde treatment. Cells which contain active GB bind 9AA and fluoresce yellow – indicated by the halo around these cells.

epithelial cells with bound Rh-I. An Olympus OM-2N camera fitted with an automatic exposure control and Kodak ASA 400 and 200 colour film was used to record our observations. All sections represent normal human colonic tissue. All 9AA stained sections (Figures 1–8, and 11–13) have magnification $\times 250$, yellow fluorescence

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

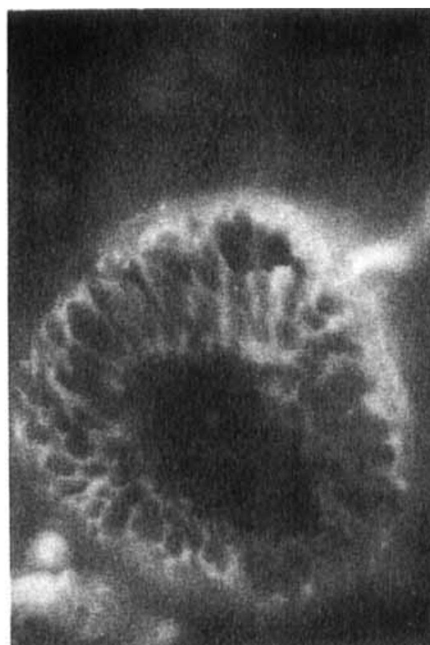


FIGURE 9 Rhodamine labeled – inhibitor treated sections. Rh-I located the GB on the surfaces of epithelial cells (red fluorescence). Magnification × 500 (see colour plate at rear).

represents cells with active GB, blue–green appearance of cells indicates lack of active GB.

RESULTS AND DISCUSSION

In this study 84 sections were examined obtained from 6 different normal colonic tissues. Data are presented from a series of frozen sections obtained from a single normal colonic tissue, in order to simplify the presentation and comparison of colour photographs. This series is representative of the whole study and summarises our observations.

In general, cells possessing active GB, bind 9AA and fluoresce yellow whereas cells in which GB is latent in the form of an enzyme-inhibitor complex (E-I) fail to bind 9AA and do not fluoresce yellow but appear pale blue–green under the microscopic conditions used. These cells with inhibited GB can be made to regain 9AA binding capacity by formaldehyde treatment[16] resulting from the dissociation of the inhibitor and consequent availability of the active centre of GB for binding 9AA. The understanding of the present study requires that we present data on the binding of 9AA to these normal colonic epithelial cells even though similar data has been presented elsewhere[18] in connection with iso-enzymes of GB.

The binding of 9AA to the cell surface of typical epithelial cells in the crypts of these frozen colonic sections is presented in Figure 1. The epithelial cells exhibit typical yellow fluorescence whilst the surrounding connective tissue and muscle cells appear

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

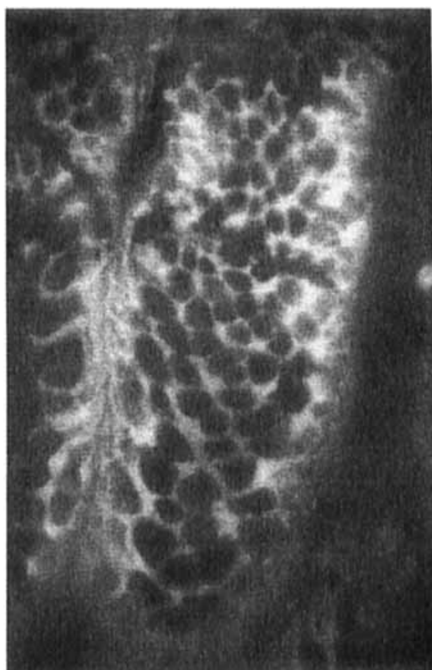


FIGURE 10 Rhodamine labelled – inhibitor treated sections. Rh-I located the GB on the surfaces of epithelial cells (red fluorescence). Magnification × 500 (see colour plate at rear).

blue green, as do most of the lymphocytes located between the crypts in this section. Pretreatment with 10 μl isotonic saline of a similar frozen section prior to 9AA staining (Figure 2) results in failure of the epithelial cells to bind 9AA, and these cells now lack yellow fluorescence and appear pale blue-green like the background. The result shown in Figure 2 could have been due to, (a) the loss or destructon of GB during exposure to 10 μl isotonic saline, or (b) to the solubilisation of a cytoplasmic inhibitor which reacted with the cell surface GB to form an E-I complex or latent GB. Further treatment of the section shown in Figure 2 with formaldehyde[16] and subsequent 9AA staining (Figure 3) demonstrated the regain in ability to bind 9AA and exhibition of cell surface yellow fluorescence. The evidence of Figure 3 indicates that the GB associated with the cell in Figure 2 was latent GB, in an E-I complex dissociable by formaldehyde treatment.[16] The data presented in Figures 1–3 demonstrates that the normal colonic epithelial cells possess a cell surface protease GB in an active from which can be recognised by inhibitors, readily extracted from the cytoplasm of the cells in the frozen section. In order to avoid the possible complication of these soluble cytoplasmic inhibitors, we employed *protected* sections[17] as the targets for potential inhibitor analysis. These *protected* sections lack cytoplasmic inhibitors and when exposed to 9AA, the epithelial cell surface GB binds the fluorescent probe and the cells appear yellow (Figure 4). We now need a technique to capture the E-I complex that can be formed on *protected* sections.

Fibrin fibrils have a high affinity[4] for PA and the binding of PA to fibrin results in a massive increase in reactivity toward plasminogen. We used *protected* sections of

RIGHTSLINK

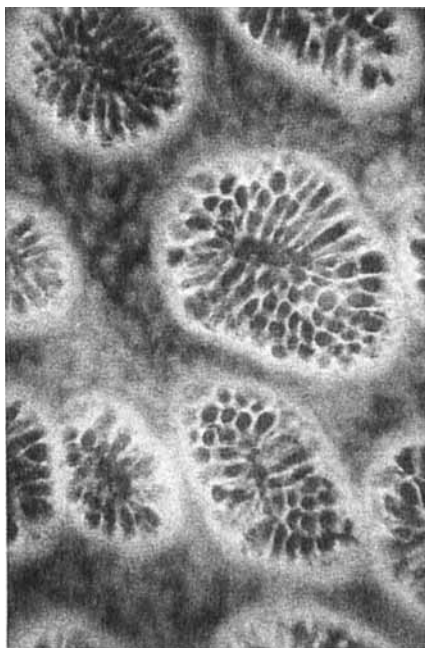


FIGURE 11 MUGB/9AA staining. The presence of MUGB blocks the binding of 9AA to the active centre of GB on epithelial cell surfaces (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

colon with active GB on the epithelial cell surfaces (Figure 4) and overlaid these sections with fibrin. After removal of the fibrin clot, these fibrin-treated *protected* sections were stained with 9AA and no yellow fluorescence was observed (Figure 5). Subsequent treatment with formaldehyde and restaining with 9AA failed to regenerate active GB (Figure 6). This observation is compatible with the concept that fibrin would detach GB from cell surfaces since GB and PA appear to be functionally similar proteases.[13] A second possibility, that the thrombin present in the formation of the fibrin overlay had attacked the cell surface GB, was excluded by demonstrating that *protected* cell GB was not affected by exposure to thrombin in the absence of fibrin (data not presented). Addition of plasminogen to the fibrin resulted in clot lysis which indicated that the GB (or PA) was still functional but was associated with the fibrin rather than the protected cell surface. It can be concluded that fibrin detached the GB from the cell surfaces in the *protected* sections.

The removal of GB from the cell surface by fibrin (Figures 5 and 6) suggested that latent GB might also be removed from *protected* cell surfaces by exposure to fibrin. Such a mechanism might provide a source of the inhibitor protein. We therefore used the direct inhibition of cell surface GB by 10 μl isotonic sodium chloride to provide membrane bound GB fully saturated with cytoplasmic inhibitor molecules (essentially providing cells shown in Figure 2 by adding inhibitor to cells typified by those in Figure 1). These sections containing E-I complex were then extensively washed in a tank of fresh isotonic saline (200 ml) to remove all the extractable cytoplasmic contents but leaving the enzyme inhibitor complex on the cell surfaces (see Figure 2).

RIGHTSLINK

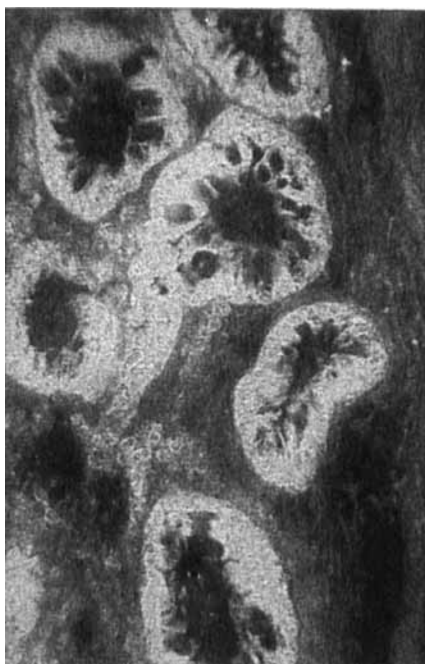


FIGURE 12 Displacement of MUGB by prolonged 9AA staining. The active centre of GB contained MUGB (Figure 11) which could be displaced by prolonged exposure to 9AA in the absence of MUGB. The cells now fluoresce yellow (compare Figure 11). The data indicates competition for the active centre of GB by MUGB and 9AA (see colour plate at rear).

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

These sections were then exposed to fibrin and the fibrin spun down in the centrifuge to release a clear supernatant fraction which should include the inhibitor proteins detached from the EI on the cell surface by the fibrin clot. The supernatant fraction was passed over a PD-10 column and the protein fraction applied to the surface of *protected* sections of normal colon. The GB on these cells was then shown to be inhibited (Figure 7) by the inability of the cells to bind 9AA, a condition reversed by treatment with fromaldehyde (Figure 8). The significance of Figures 7 and 8 is that they demonstrate the transfer of inhibitor protein from the cells in one section (the donor) to the GB on *protected* cells (in the target section) and that this transfer has been mediated by the binding of GB (from E-I) to the fibrin. In binding the GB, the fibrin first dissociated the cell surface E-I complex from the cell. Second, by binding the enzyme to the fibrin, the inhibitor has now been released into the soluble phase. The arrival of the transferred inhibitor on the cell surface GB is signalled by the inhibition of GB in Figure 7 and its regeneration in Figue 8. A flow diagram outlining the events illustrated in Figures 1–8 is presented in Scheme 1.

We attempted to confirm the conclusion that the cytoplasmic inhibitor can be transferred to fibrin from the cell membrane bound latent enzyme-inhibitor complex by direct visualisation of the transferred inhibitors. A rhodamine-labelled protein fraction (Rh-I, see Methods), was applied to *protected* frozen sections of normal colon. The Rh-I, (a) selectively located the surface of colonic epithelial cells (Figures

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

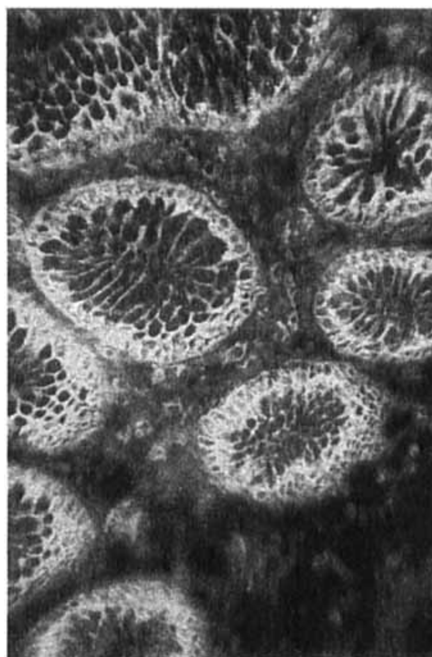


FIGURE 13 The presence of MUGB protects GB from the cytoplasmic inhibitor of GB 10 μl isotonic saline in the presence of MUGB fails to inhibit the cell surface GB, which is still capable of binding 9AA and fluoresces yellow. (Compare with Figure 2 in which MUGB was omitted) (see colour plate at rear).

9 and 10), (b) blocked the binding of 9AA to these cells (data similar to Figure 2) and, (c) was displaced by formaldehyde treatment with regain of cell surface binding of 9AA (data similar to Figure 3). We conclude that the fibrin fibrils dissociated the epithelial cell surface bound E-I, with release of extractable I, and that the latter could be labelled with rhodamine without abolishing its ability to recognise cell surface GB in *protected* sections. The Rh-I behaved as unlabelled inhibitor but had the advantage that the binding of Rh-I to the cell membrane GB could be monitored by fluorescent microscopy (Figures 9 and 10).

We have taken advantage of the known affinity of fibrin for PA and shown that fibrin also has a high affinity for cell surface bound GB and latent GB (Figures 7 and 8). The functional similarity[13] between PA and GB has again been confirmed in this study using a common affinity for fibrin. In view of the report[15] that GB and PA are different enzymes, (based upon monoclonal antibodies directed to PA) it is worth mentioning the following points. The active site titrants *p*-nitrophenyl-4-guanidinobenzoate (NPGB),[19] and 4-methylumbelliferyl-4-guanidinobenzoate (MUGB)[20] were designed as inhibitors of seryl proteases; they have proved to be highly successful in the inhibition and assay of most trypsin-like enzymes in solution. Two notable exceptions amongst trypsin-like enzymes are GB[9] and single chain t-PA.[21] The enzymic properties of a protease (e.g. t-PA) bound to a cell surface receptor may be different from the enzymic properties of t-PA in free solution or associated with insoluble fibrin fibrils. The interaction of MUGB with the active centre of GB on colonic epithelial

RIGHTSLINK

cells can be followed by competitive binding studies with 9AA. The data presented below will indicate that MUGB does not irreversibly inhibit cell surface GB but acts as an active site directed competitor for this site with, (a) 9AA and, (b) the cytoplasmic protein inhibitors of GB. *Protected* sections of colonic tissue treated with MUGB/9AA result in the labelling of GB with 9AA (data similar to Figure 4). This indicates that 9AA remains bound to the active centre and blocks the arrival of non-fluorescent MUGB when the two agents are offered simultaneously as a first choice. MUGB treatment of *protected* sections followed by MUGB/9AA staining demonstrated the binding to the active centre of GB on these cells by MUGB which blocked the subsequent binding of 9AA present in the MUGB/9AA solution (Figure 11). Prolonged exposure to 9AA resulted in the binding of 9AA and reappearance of yellow fluorescence (Figure 12). The data in Figures 11 and 12 can be interpreted as follows. MUGB is attracted to the active centre of GB as an active site directed substrate being cleaved to MU just as has been described for GB in solution.[9] When 9AA with MUGB is introduced to this section, the MUGB has a higher affinity for GB and 9AA does not bind (Figure 11). The 9AA only binds when this section is subsequently placed in 9AA lacking MUGB (Figure 12) and the residual MUGB associated with the active centre has been either competitively displaced by the higher concentration of 9AA or the MUGB has been cleaved by the GB and subsequently released as products. This evidence would indicate that MUGB and 9AA are both attracted to the same binding site on these cell surfaces, viz the active centre of GB.

It has previously been shown that 9AA can be used to *protect* the GB on epithelial cell surfaces from inhibition by cytoplasmic proteins extracted from these frozen sections in 10 μl isotonic saline placed on top of the section,[12] (see Figures 1–4 above). If MUGB has a high affinity for the active site of GB on epithelial cell surfaces, MUGB should also offer protection from interaction of cell surface GB with the cytoplasmic inhibitors. This postulate can readily be verified by placing 10 μl of isotonic saline containing MUGB on frozen sections and following the course of reaction with the fluorescent probe 9AA. If it is considered what would happen if the cytoplasmic inhibitor bound to the GB in the presence of MUGB, then after 9AA staining, the epithelial cell surfaces would appear blue-green, since no 9AA would bind. On the other hand, if the presence of the MUGB prevented the binding of cytoplasmic inhibitor, the epithelial cell surfaces would be capable of binding 9AA. In fact the presence of MUGB did protect the active centre of the GB from approach by the cytoplasmic inhibitor (Figure 13). We conclude that the enzyme which binds 9AA also has a high affinity for MUGB and this enzyme also has a high affinity for fibrin fibrils. The GB of the enzyme-inhibitor complex (latent GB on cell surfaces) also has a high affinity for fibrin resulting in the transfer of cell surface proteins to fibrin and release of potent inhibitor protein capable of recognising GB on *protected* sections. We do not know whether GB and PA are identical but they are certainly very similar in their recognition by protein inhibitors of proteases and low molecular weight active site directed agents such as MUGB, NPGB, 9AA and Dans GGCK.

The present study has shown that the affinity of fibrin for GB (and or PA) can be used to prepare cytoplasmic inhibitor proteins which recognise the cell surface GB associated with colonic epithelial cells. It may well be that *in vivo* the status of cell surface GB can be modified not only by the release of inhibitors from the same cell, but by the contact of fibrin fibrils in the extracellular matrix or in the blood vessels. Thus latent GB on the surface of cells could give rise to fibrin associated active protease, which no longer combines with the inhibitor for which it once had a high

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

RIGHTSLINK

affinity. These possibilities could have profound biological consequences in terms of fibrinolysis and metastatic activity of certain cells.

Acknowledgement

FSS gratefully acknowledges the generous financial support given by the Wigan and District Cancer Research Committee/J.T. Starkey Memorial Fund, without which this study would not have been carried out in Manchester.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/15/11
For personal use only.

References

1. Kruithof, E.K.O. (1988) *Enzyme*, **40**, 113–121.
2. Mangel, W.F. (1990) *Nature (Lond.)*, **344**, 488–489.
3. Stryer, L. (1988) "Biochemistry", Third Edition, p. 256. New York: Freeman and Company.
4. Hoylaerts, M., Rijken, D.C., Lijnen, H.R., and Collen, D. (1982) *J. Biol. Chem.*, **257**, 2912–2919.
5. Vassali, J.D., Baccino, D. and Belin, D. (1985) *J. Cell Biol.*, **100**, 86–92.
6. Moscatelli, D. and Rijkin, D.B. (1988) *Biochim. Biophys. Acta*, **948**, 67–85.
7. Mullins, D.E. and Rohrlich, S.T. (1983) *Biochim. Biophys. Acta*, **695**, 177–214.
8. Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) *Adv. Cancer. Res.*, **44**, 139–266.
9. Steven, F.S. and Al-Ahmad, R.K. (1983) *Eur. J. Biochem.*, **130**, 335–339.
10. Steven, F.S., Griffin, M.M. and Al-Ahmad, R.K. (1985) *Euro. J. Biochem.*, **149**, 35–40.
11. Steven, F.S., Jackson, H., Jackson, N.C. and Wong, T.L.H. (1987) *Brit. J. Cancer*, **55**, 29–32.
12. Steven, F.S. and Booth, N.A. (1991) *J. Enz. Inhib.*, **4**, 273–279.
13. Steven, F.S., Griffin, M.M., Cederholm-Williams, S.A., Mangel, W.F. and Maier, H. (1991) *Anticancer Res.*, (in press).
14. Kettner C. and Shaw, E. (1981) *Meth. Enzymol.*, **80**, 826–842.
15. Perry, J.K. and Scott, K.G. (1990) *Bioscience Reports*, **10**, 469–472.
16. Steven, F.S., Maier, H. and Arndt, J. (1989) *J. Enz. Inhib.*, **3**, 145–157.
17. Steven, F.S. and Griffin, M.M. (1991) *J. Enz. Inhib.*, **5**, 77–85.
18. Steven, F.S., Griffin, M.M., Maier, H. and Talbot, I.C. (1990) *Biol. Chem. Hoppe Seyler*, **371**, Suppl., pp. 89–94.
19. Chase, T. and Shaw, E.N. (1967) *Biochem. Biophys. Res. Commun.*, **29**, 508–514.
20. Coleman, P.L., Latham, H.G. and Shaw, E.N. (1976) *Meth. Enzymol.*, **45**, 12–26.
21. Geiger, M. and Binder, B.R. (1987) *Biochim. Biophys. Acta*, **912**, 34–40.

RIGHTSLINK