*J. Enzyme Inhibition*, 1990, Vol. 4, pp. 63–73
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# COMPETITIVE INHIBITION OF A TUMOUR CELL SURFACE PROTEASE. A RAPID TECHNIQUE FOR *IN VITRO* TESTING OF SELECTIVE TARGETING SYSTEMS

F.S. STEVEN*†, M.M. GRIFFIN†, F.B. BARNETT‡ and A.A. EPENETOS§

†*Department of Biochemistry and Molecular Biology*, ‡*Department of Cell and Structural Biology, School of Biological Sciences, University of Manchester, Manchester, M13 9PT, UK.* §*Imperial Cancer Research Fund Oncology Group, Royal Postgradute Medical School, Hammersmith Hospital, London, W12 0HS, UK.*

*(Received 9 January 1990)*

The active centre of a protease on the surface of tumour cells can be located by its affinity for an active site – directed inhibitor, 9-amino acridine. Cells which have uninhibited proteases, bind 9-amino acridine and fluoresce in resin sections. The leukaemic rat was used as a model system to provide tumour cells in a well defined location. Drugs when coupled to a ligand (directed to the active centre of the protease) compete for this binding site with 9-amino acridine. Thus, competitive inhibition of the tumour cell surface protease provides a rapid technique for demonstrating the delivery of liganded molecules to the surface of tumour cells *in vitro*.

KEY WORDS: Protease, tumour cell, targeting, 9-aminoacridine, guanidinobenzoatase, mitomycin C.

## INTRODUCTION

Tumour cells possess the protease guanidinobenzoatase (GB).[1] GB occurs on the cell surface either as active GB or as latent GB; the latter being an enzyme-inhibitor complex,[2] which can be dissociated either by formaldehyde treatment or by acetone treatment.[3] The presence of active GB on tumour cells could be used to target cytotoxic drugs and other compounds to these cells by taking advantage of competitive inhibition equilibria.[4] We have explored this possibility with the cytotoxic agent[5] mitomycin C and the potential chelating agent DTPA (diethylene triamino pentaacetic acid).[6] This chelating agent has the potential for radio labelling and possible use in tumour location *in vivo*.[7]

In order to test the successful *in vitro* targeting of these compounds to the cell surface GB, resin sections of leukaemic rat kidneys were employed. The leukaemia cells in these sections contained active GB which could be located with 9-amino acridine (9-AA); the latter is a competitive inhibitor[1] of GB and binds to the active centre of this protease.[1] Competition experiments, in which the liganded mitomycin C (MMC*) or the liganded chelator (LC*) were shown to compete for the active centre of GB with 9-AA demonstrated the successful targeting of these new com-

*Correspondence.



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pounds to the tumour cell surface by means of their affinity for GB. The results indicate that the principle of competitive inhibition can be used to target cells with active GB and this in turn may lead to both therapy and location technqiues *in vivo*. In the mean time, the leukaemic kidney acts as a useful *in vitro* model, since competitive inhibition reactions involving GB in our study (and possibly other enzymes) can be rapidly screened using fluorescent microscopy techniques.[8]

We first outline the synthetic steps used in making MMC* and LC,* followed by the procedure used for the location of pure product. We then describe the location of GB on leukaemia cells, employing resin sections and the yellow fluorescent probe 9-AA which binds to the active centre of GB. Competitive experiments with MMC* and LC* in the presence of 9-AA demonstrate the affinity of all three reagents for the active centre of GB on leukaemia cells. We suggest that this principle, based on competitive inhibition reactions, could have wider applications in the design and testing of targeted delivery systems employing other cell surface enzymes.

## MATERIALS

9-Amino acridine, agmatine, succinic anhydride, 1,1′ carbonyl diimidazole, *N*-hydroxy sulphosuccinimide, S-Sepharose and diethylene triaminopentaacetic acid (DTPA) anhydride were purchased from Sigma Chemical Co. Ltd., St. Louis, Mo, USA. Mitomycine C was kindly provided as a gift by Macarthy Medical, Farillon House, Ashton Rd., Romford, Essex, RM3 8UE, UK.

Fresh rat T-cell lymphoblastic leukaemia[9,10] cells were kindly provided by Dr. H. Jackson of the Department of Physiological Sciences, University of Manchester.

## METHODS

### *Preparation of MMC**

The object was to obtain a pure sample of agmatine (**I**) linked through its amino group to a succinyl spacer linked to the amino group of mitomycin C. this complex will be referred to as MMC* (Figure 1). The synthetic approach was to make an excess of succinylated agmatine (**II**) (Reaction 1) which could then be condensed with a limited amount of Mitomycin C in the presence of a carbodiimide to produce the product (**III**) or MMC* in Figure 1 (Reaction 2).

The first synthetic step was to prepare and purify succinylated agmatine. We used 20 mg agmatine in 20 ml sodium bicarbonate buffer, pH 8.0, and added 2 g succinic anhydride whilst maintaining the pH within the range pH 7–8 for 6 h. The excess succinic anhydride was used to ensure complete conversion of the agmatine to the succinylated derivative, this excess reagent rapidly hydrolysed to succinic acid and was later removed as a waste product.

The product (**II**) was collected by passing the reaction mixture over a column of sulphated Sepharose; the product (**II**) being bound to the column, whilst the succinic acid was not. The column was washed with 500 ml distilled water and then the succinylated agmatine (**II**) was eluted at pH 11.5. The pH of the eluate was adjusted to 7.0 and the presence of succinylated agmatine (**II**), as the only product, was demonstrated by paper chromatography employing n-butanol:acetic acid:water

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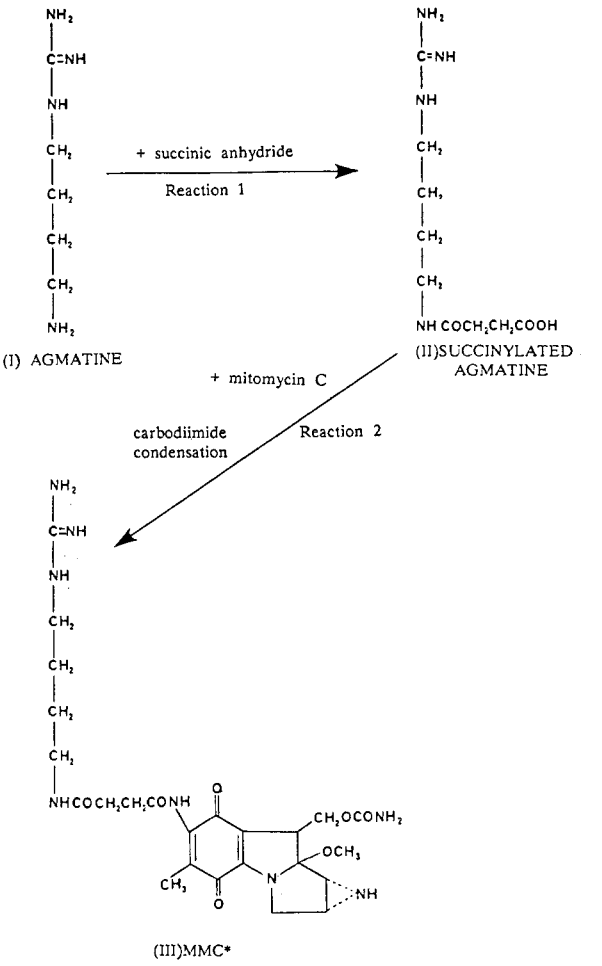


FIGURE 1 Synthesis of MMC* from mitomycin C, agmatine and succinic anhydride.

(8:1:1) as solvent. The presence of a new Sakaguchi positive compound with Rf distinct from the parent agmatine (Figure 2) confirmed the synthesis of succinylated agmatine (**II**) as the only product.

The second step required the condensation of the free carboxyl group of (**II**) with mitomycin C by means of carbodiimide coupling with the only free amino group of mitomycin C to form product (**III**) or MMC*. The mitomycin C was introduced at this the last step so as to protect its structure from reactions which might have taken place in the presence of the succinic anhydride used in the first step. We used 4 mg mitomycin C added to the succinylated agmatine at pH 7.0 in the presence of excess carbodiimide (1,1′ carbonyl diimidazole) together with added excess of *N*-hydroxy sulphosuccinimide.[11] The latter reagent was added to promote carbodiimide condensation.[11] The condensation reaction was allowed to proceed at room temperature in the dark for 18 h.

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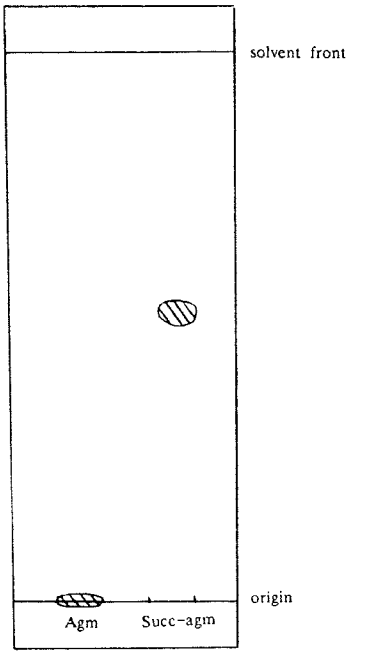


FIGURE 2 Paper chromatogram of agmatine and succinylated agmatine, (Butanol: acetic acid: water, 8:1:1) stained with Sakaguchi reagents.

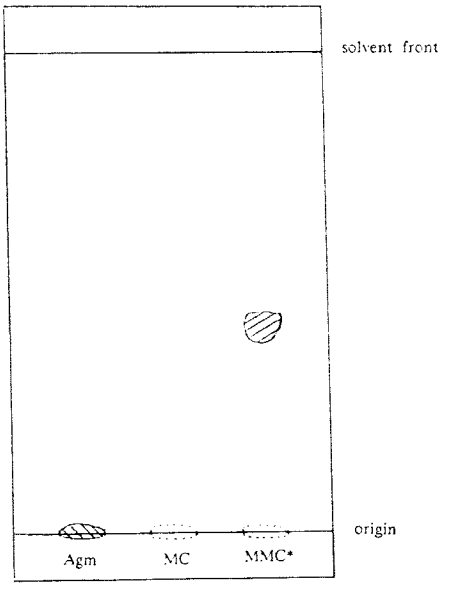


FIGURE 3 Paperchromatogram of agmatine, mitomycin C and MMC*, (Butan acid: water, 8:1:1) stained with Sakaguchi reagents.

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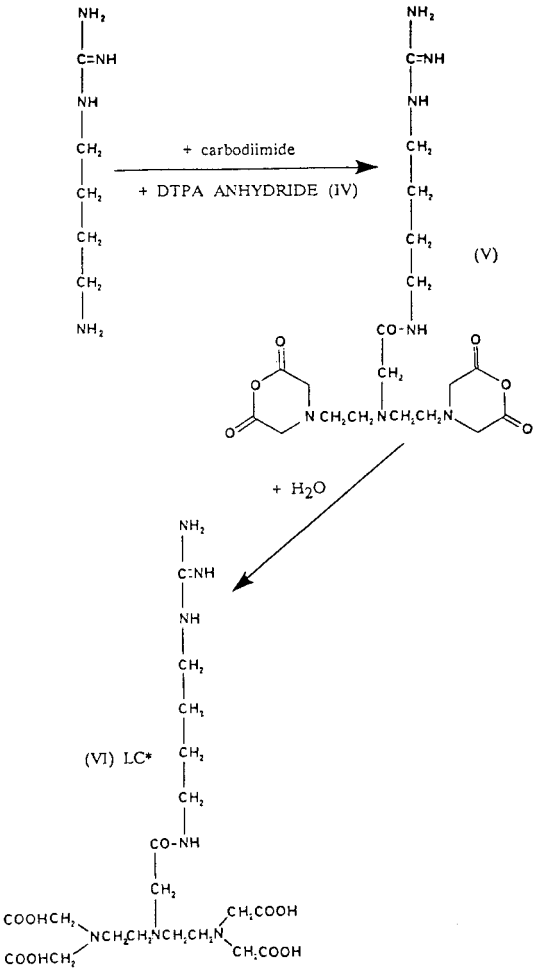


FIGURE 4 Synthesis of LC* from agmatine and DTPA.

TLC analysis and paper chromatographic analysis demonstrated that the carbodiimide promoted condensation (Reaction 2) resulted in a new fluorescent derivative of mitomycin C in good yield (Figure 3). Sakaguchi staining[12] of the paper chromatographic separations demonstrated that this new fluorescent product of mitomycin C possessed a guanidino group and must therefore be the product (**III**) or MMC*. This new product gave an intense red stain with the Sakaguchi reagent; we used this test as a marker for the identification of our product (**III**) when we used many paper chromatographic separations to prepare pure MMC* for further study. The MMC* eluted from several paper chromatograms was freeze dried and dissolved in 1 ml isotonic saline for fluorescent microscopic studies (see later). Assuming the complete conversion of the 4 mg mitomycin C used in this preparation, the solution used for fluorescent labelling of tumour cells contained approximately $1.2 \times 10^{-6}$ M MMC*.

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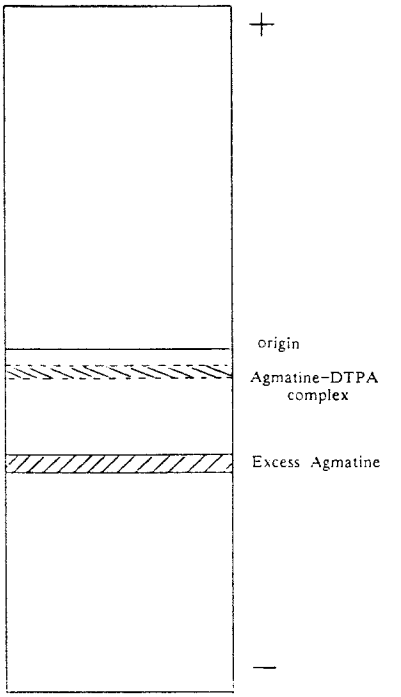


FIGURE 5 Paper electrophoresis strip of agmatine - DTPA complex run in pyridine acetate buffer, pH 6.1.

*Preparation of Liganded Chelator (LO*) or Diethylene Triaminopentaacetic Acid-Agmatine Complex*

In theory, DTPA could be linked through any of its five free carboxyl groups to agmatine via carbodiimide condensation. We chose to use the cyclic anhydride of DTPA and coupled this to agmatine prior to hydrolysis of the two acid anhydride moieties. Under these conditions of condensation the site of peptide bond formation is defined and the resultant tetra acetic acid complex retains potential binding sites for metal chelation. The synthetic strategy is shown in Figure 4.

Equimolar amounts ($10^{-3}$ M) of DTPA anhydride (**IV**, Figure 4) and agmatine were dissolved in dimethyl sulphoxide and excess carbodiimide added to promote peptide bond formation (**V**, Figure 4). The mixtuer was stirred for 18 h and then 200 ml of water added to the organic solvent to hydrolyse the anhydride and produce the potential chelating agent (**VI**), referred to as the liganded chelator (LC*).

The aqueous layer was concentrated by rotary evaporation and subjected to paper electrophoresis at pH 6.1 in pyridine acetate buffer with agmatine as a standard. The presence of LC* was detected on the paper by Sakaguchi staining; a strong red zone (Figure 5) having markedly less positive charge than free agmatine due to the substitution of the α-amino group with the DTPA moiety (Figure 4, Structure **VI**). The LC* band was cut out from the electrophoresis strip and eluted in water. It was then freeze dried and used for inhibition studies of GB on leukaemia cells. Approximately $10^{-6}$ M LC* was employed as a stock solution in these inhibition studies.

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*Competitive Inhibition Studies on GB Associated with Rat Leukaemia Cells in Resin Sections*

Formaldehyde fixed rat leukaemic kidney tissue in historesin sections (5 $\mu$m) has previously been used to demonstrate the fluorescent location of cells possessing active GB.[8] In this study we used acetone to dehydrate the leukaemic kidney tissue prior to embedding in historesin.[8] The acetone had the added advantage of displacing the protein inhibitor from the leukaemia cell surface GB leaving the enzyme in an active from which binds 9-AA. The leukaemia cells in these sections lie in the intertubular spaces and within the glomeruli. The leukaemia cells can easily be recognised by their location, their morphology and their ability to bind 9-AA to the active centre of GB.[8] The leukaemia cells in these sections provide an ideal testing ground for experiments designed to target cells possessing active GB (*in vitro*) by means of competitive inhibition.

*Direct Staining with 9-AA*

We have observed with resin sections that the time required for equilibration of GB and 9-AA needs to be increased from 2 to 10 min. The sections were placed in 9-AA, $10^{-3}$ M, 200 $\mu$l, for 10 min. prior to washing with isotonic saline for 1 min. When these sections were examined using the Leitz Diaplan fluorescence microscope with barrier filter K 490 and filter cube G (ref. no. 513602), the cells possessing active GB exhibited a bright yelow fluorescence.

*Competitive inhibition of GB with BZAR [$(CbzArgNH)_2$Rhodamine*[13]

The sections were first treated with 200 $\mu$l BZAR, $10^{-5}$ M for 30 min prior to 9-AA staining as described above. Reversal of BZAR inhibition of GB[14] was demonstrated by prolonged exposure to 9-AA ($10^{-3}$ M, 500 ml, 18 h). This treatment effectively reduced the concentration of BZAR to an insignificant level whilst maintaining the concentration of 9-AA at $10^{-3}$ M for 18 h. As a result, 9-AA replaced the BZAR in the active centre of GB on the leukaemia cells.

*Competitive Inhibition of GB with MMC* and LC**

Resin sections of leukaemic kidney tissue were exposed to 200 $\mu$l of approximately $10^{-6}$ M MMC*, or LC*, for 30 min prior to 9-AA staining as described above. The subsequent displacement of MMC* or LC* from the active centre of leukaemia cell GB was achieved by prolonged exposure to 9-AA ($10^{-3}$ M, 500 ml, 18 h).

## RESULTS AND DISCUSSION

*Fluorescent Studies on Rat Leukaemia Cells in Kidney Sections*

Direct 9-AA staining of the kidney sections clearly showed the presence of the leukaemia cells fluorescing yellow due to 9-AA bound to the cell surface GB, in the intertubular spaces and within the glomeruli (Figure 6). Prior exposure of these kidney sections to BZAR, followed by 9-AA, demonstrated the inhibition of GB on

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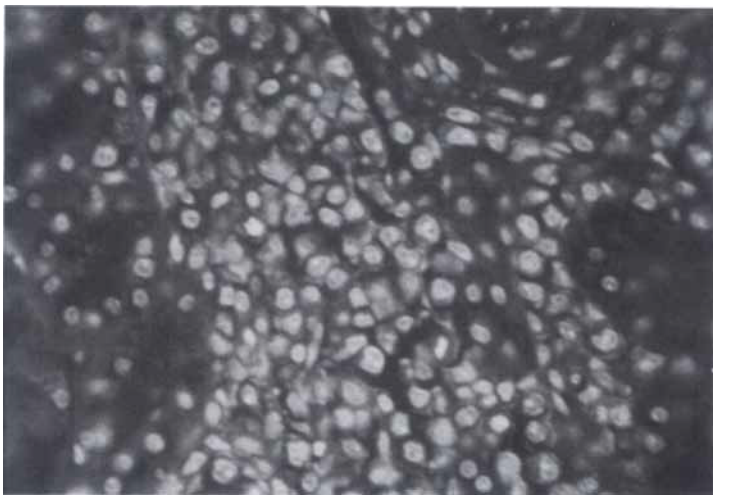

FIGURE 6 Resin sections of leukaemic kidney after direct staining with 9-AA. The leukaemia cells possess active GB, bind 9-AA and fluoresce yellow. The leukaemia cells are located mainly in the intertubular spaces; the tubules appear green. Magnification × 500. (See colour plates at back of issue).

the leukaemia cell surfaces; the cells no longer bound 9-AA and remained virtually unstained by the fluorescent yellow marker for GB (Figure 7). After prolonged exposure to 9-AA ($10^{-3}$ M, 18 h) the BZAR was slowly displaced and 9-AA was again able to bind to the leukaemia cells with the consequent regain in yellow fluorescence seen on these cells (similar to Figure 6).

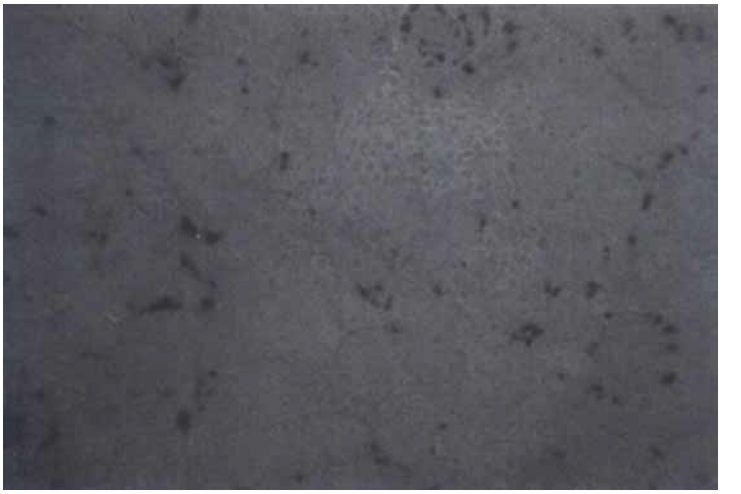

FIGURE 7 Resin sections of leukaemic kidney treated first with BZAR and then with 9-AA. The leukaemia cells lack active GB and fail to fluoresce yellow (compare with Figure 6). The outline of the tubules and leukaemia cells within the intertubular spaces can just be distinguished. The BZAR has blocked the active centre of GB, preventing the binding of 9-AA. Magnification × 250. (See colour plates at back of issue).

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FIGURE 8 Resin sections of leukaemic kidney first treated with MMC* and then with 9-AA. The leukaemia cells do not bind 9-AA and fail to fluoresce due to the binding of MMC* at the active centre of GB. MMC* behaves similarly to BZAR (Figure 7). Magnification × 500. (See colour plates at back of issue).

The above experiments demonstrate that 9-AA binds only to cells possessing active GB (Figure 6) and after selective inhibition of this enzyme the leukaemia cells fail to bind 9-AA. Since the process was reversed by prolonged exposure to 9-AA (500 ml, $10^{-3}$ M, 18 h) this is a reversible competitive inhibition. These experiments define the rules and interpretation of competitive experiments employing non-fluorescent liganded complexes which also interact with the active centre of GB on leukaemia cells.

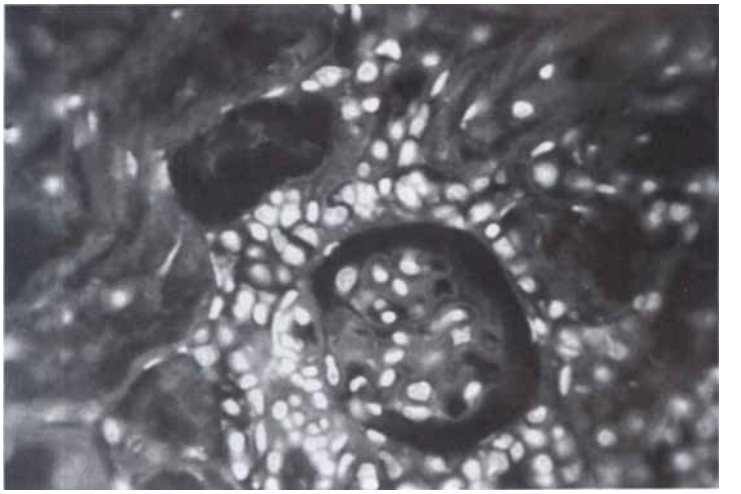

FIGURE 9 Resin sections of leukaemic kidney first treated with MMC* and then prolonged exposure to 9-AA. The leukaemia cells which originally failed to bind 9-AA, due to MMC* bound to the active centre, have now regained their ability to bind 9-AA and fluoresce yellow. The 9-AA has displaced the MMC* from the active centre of GB on the cell surface of the leukaemia cells. Magnification × 500. (See colour plates at back of issue).

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FIGURE 10 Resin section of leukaemic kidney first treated with LC* and then with 9-AA. The leukaemia cells can just be seen due to their very faint yellow surface staining with 9-AA. Almost all the GB on the leukaemia cells has bound LC* at the active centre and fails to bind 9-AA. Magnification × 500. (See colour plates at back of issue).

Although MMC* is fluorescent in aqueous solution and also when bound to tumour cells in frozen sections, MMC* was not found to fluoresce in resin sections when examined with filter K 490 and filter cube G. These are the fluorescent conditions most suited to reveal 9-AA in resin sections. Pretreatment of resin sections with MMC* ($10^{-6}$ M, 30 min) prior to 9-AA ($10^{-3}$ M, 10 min) resulted in complete

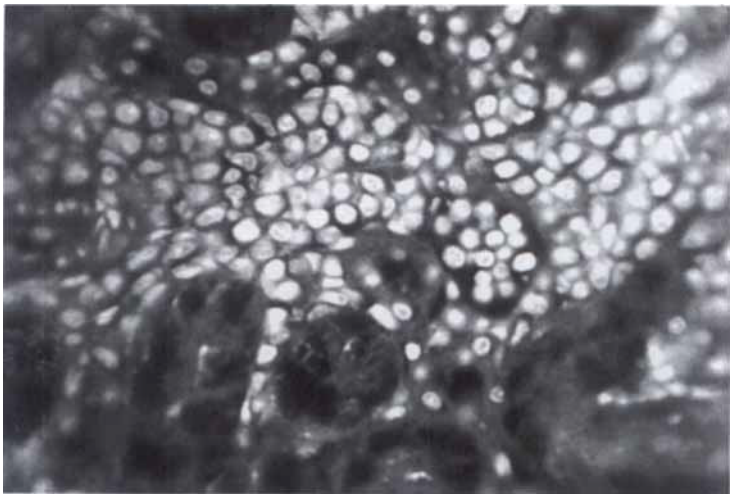

FIGURE 11 Resin sections of leukaemic kidney first treated with LC* and then prolonged exposure to 9-AA. The leukaemia cells bind 9-AA and fluoresce yellow. The inhibiting LC* has been displaced from the active centre of GB on the leukaemia cells by prolonged exposure to excess 9-AA in an exchange of molecules which have an affinity for the active centre of GB. This exchange can be followed by fluorescent microscopy since 9-AA is highly fluorescent and LC* is not fluorescent at all. Magnification × 500. (See colour plates at back of issue).

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inhibition of yellow fluorescent labelling of leukaemia cells (Figure 8). The bound MMC* prevented the cell surface GB from binding 9-AA. Prolonged immersion of this GB inhibited section in 9-AA for 18 h resulted in the displacement of MMC* by 9-AA and the consequent restaining of the leukaemia cells (Figure 9). Thus the MMC* behaved like BZAR in this competition with 9-AA for the active centre of GB. In exactly the same way, the GB labelled with 9-AA will lose this 9-AA when placed in a tank of 200 ml fresh isotonic saline for 3–4 h. Clearly these reagents are all exchangeable and compete for the active centre of GB in a concentration-dependent manner. The importance of the binding of MMC* to the active centre of tumour cell surface GB will be discussed later. At present it is enough to demonstrate the targeting of mitomycin C via liganded MMC* to the leukaemia cell GB.

The potential chelating agent (LC*) also inhibited GB on leukaemia cell surfaces (Figure 10), since subsequent staining with 9-AA failed to locate the tumour cells by yellow fluorescence. The LC* was displaced by prolonged exposure to 9-AA ($10^{-3}$ M, 500 ml 18 h) (Figure 11). Clearly the MMC*, LC*, BZAR and 9-AA are all exchangeable at the same binding site; viz. the active centre of GB on the leukaemia cell surface. The data show that each of these agents is selectively bound to GB-containing cells and that the agmatine moiety of both MMC* and LC* directs these reagents to the active centre of GB in resin sections of leukaemia kidney tissue.

The present paper defines the chemistry and competitive potential of MMC* and LC* for tumour bound GB. Further studies will be directed to the biological testing of these liganded agents to determine whether or not MMC* is more effective than mitomycin C alone in tumour repression experiments and whether LC* can be used to carry radioactive metals to secondary tumours in nude mice.

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## *Acknowledgement*

FSS wishes to thank the Imperial Cancer Research Fund for generous financial support without which this study could not have been carried out in Manchester. We wish to thank Dr. W.F. Mangel, Brookhaven National Laboratory, Associated Universities, Upton Long Island, New York 11973, USA, for the generous gift of BZAR used in this study.

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