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FLUORESCENT INHIBITORS OF A CELL SURFACE PROTEASE USED TO LOCATE LEUKAEMIA CELLS IN KIDNEY SECTIONS

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Guanidinobenzoatase is a trypsin-like protease on the surface of cells capable of migration, for example leukaemia cells. We have used a number of fluorescent probes that are competitive inhibitors of guanidinobenzoatase to locate leukaemia cells in resin sections of kidney tissue obtained from leukaemic rats. We have demonstrated how this competitive inhibition system can be used to direct desired molecules (such as cytotoxic drugs) to these cells and to monitor the arrival of such compounds at the active site of guanidinobenzoatase. The principles developed in this study could equally well be applied to other enzymes on other cells provided suitable competitive inhibitors were designed. The presence of an enzyme on the surface of a cell can be used to direct molecules to that cell provided that these molecules contain a functional group that acts as an inhibitor for the chosen enzyme.

KEY WORDS: Competitive inhibition, fluorescent probes, cell surface, protease.

INTRODUCTION

The proteolytic enzyme guanidinobenzoatase[1] is associated with cells capable of migration, in particular tumour cells[1,2]. Fluorescent probes that are directed to the active centre of guanidinobenzoatase have been employed to locate cells possessing this enzyme by means of fluorescent microscopy[2] using frozen sections and formalin fixed wax-embedded sections. These probes were shown to be competitive inhibitors of guanidinobenzoatase by kinetic analysis[1,2] employing the cleavage of the fluorogenic substrate 4-methylumbelliferyl-*p*-guanidinobenzoate[3] and a crude preparation of guanidinobenzoatase. One of these probes, 9-aminoacridine, has the advantage that additional molecules of the probe are able to stack on the initial molecule which binds to the active centre of guanidinobenzoatase[2,4]. This lateral aggregation of planar molecules appears to be similar to the intercalation of 9-aminoacridine in DNA. In the present study it has the advantage of increasing the fluorescent signal emitted by the cells possessing the target enzyme. The result is that cells binding 9-aminoacridine have a yellow fluorescence when illuminated with violet light[2], and the unstained cells and connective tissue appear blue. We employed a second stacking molecule, propidium iodide, to co-stack on 9-aminoacridine-treated cells in wax embedded sections[4]; this led to a change in fluorescence from a yellow to a pink colour on the

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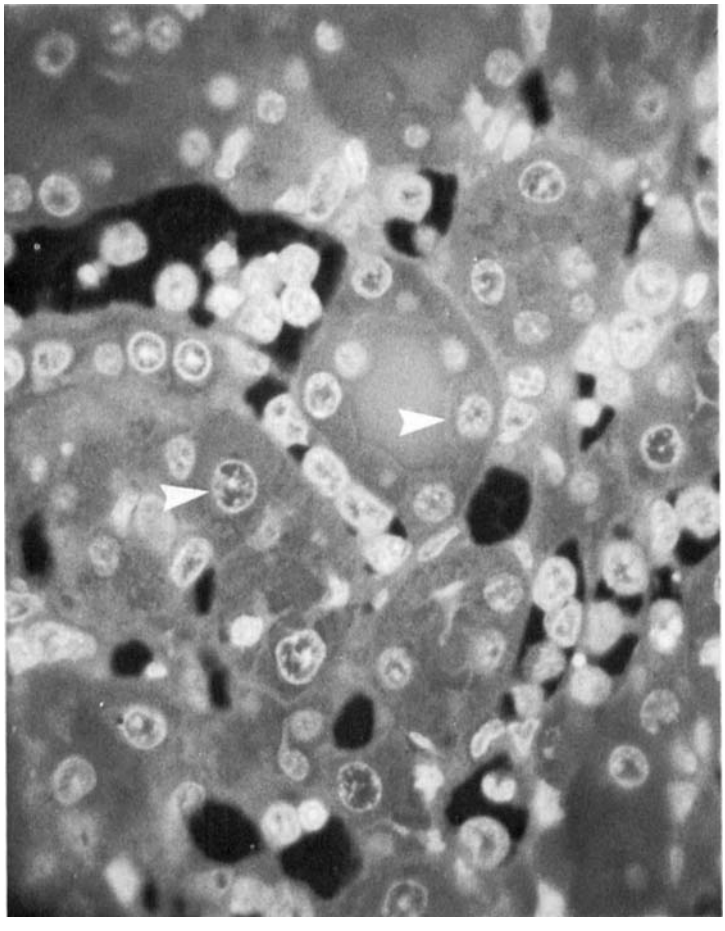

FIGURE 1† Resin section stained with 9-aminoacridine, magnification × 600. The arrowheads point to the perinuclear ring staining of tubule cell nuclei whereas the cytoplasm and cell surface of tubule cells are *not* stained. Leukaemia cells in the intertubular spaces are stained yellow over their entire surfaces. (See Colour Plate I).

surface of cells possessing the target enzyme. The advantage of this double staining procedure was the improved photographic contrast of pink on blue compared to yellow on blue[5]. This advantage was somewhat offset by the fact that the nuclei of all cells in the wax embedded sections stained red. In the case of leukaemia cells the nuclei often occupy most of the cell space and it was difficult to distinguish between the pink surface staining of guanidinobenzoatase and red nuclear staining in doubly stained wax embedded sections. We have overcome these problems by employing resin sections of glutaraldehyde-fixed tissue. In this report we illustrate the fluorescent labelling of leukaemia cells with both competititive inhibitors and also a non-competitive inhibitor of guanidinobenzoatase.

†All the figures show fluorescent microscopic pictures of wax or resin embedded formalin-fixed rat kidney tissue invaded by leukaemia cells.

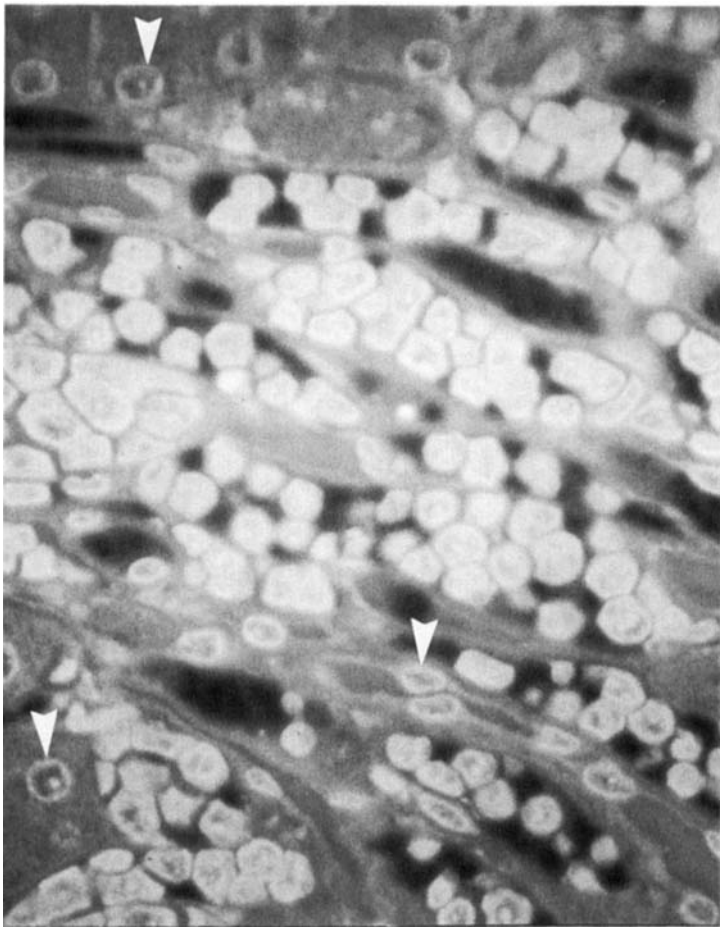

FIGURE 2 Resin section stained with 9-aminoacridine followed by propidium iodide, magnification × 600. The arrowheads point to the nuclei of tubule cells; leukaemia cells in the inter-tubular spaces are stained pink over their entire surfaces. (See Colour Plate II).

MATERIALS

Rhodamine, 9-aminoacridine and propidium iodide were purchased from Sigma Chemical Company, St Louis, Mo., USA. Dansyl-sulphaguanidine was prepared from sulphaguanidine (kindly provided by Glaxo Ltd, Greenford, Middlesex) by the method of Gray and Hartley[6]. A crude preparation of rhodamine bisguanidinobenzoate was prepared by condensing guanidinobenzoic acid and rhodamine in the presence of a carbodiimide according to the method described for the analogous synthesis of (Cbz-Arg-NH_2)$_2$-Rhodamine described by Leytus *et al.*[7]. This preparation was expected to contain both the mono- and bis-substituted rhodamines, and for the present purposes was not purified further.

The leukaemic kidneys were obtained from rats with T-cell lymphoblastic

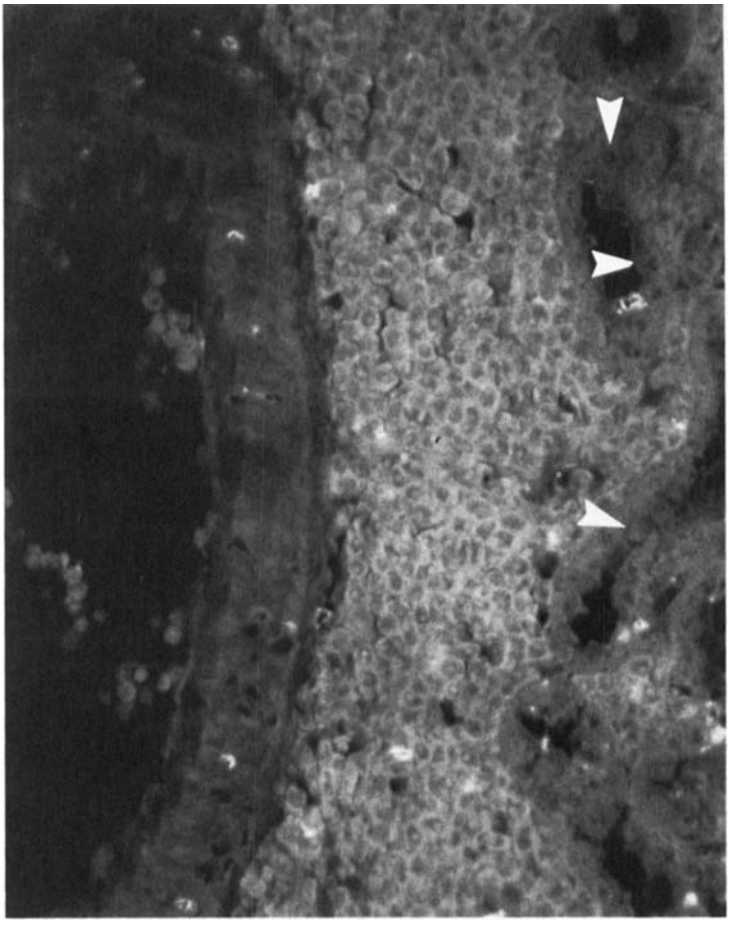

FIGURE 3 Wax section stained with 9-aminoacridine followed by propidium iodide, magnification × 300. The arrowheads point to nuclei of tubule cells which are stained completely, making these nuclei difficult to distinguish from the adjacent tumour cells which exhibit total staining of their surface. The tumour cells are seen in the intertubular spaces and also as individual leukaemia cells within a blood vessel. (See Colour Plate III).

leukaemia[8,9] and after glutaraldehyde fixation were embedded in LKB 2218-500 historesin (purchased from LKB, Bromma, Sweden). Sections were cut at 1 μm.

METHODS

Sections were stained with 9-aminoacridine (10^{-3} M) for 5 min followed by 30 s washing in isotonic saline. Double staining with propidium iodide required the 9-aminoacridine-stained sections to be stained with 6×10^{-5} M propidium iodide for 1 min followed by 10 s wash in isotonic saline prior to fluorescent microscopy. Dansyl sulphaguanidine (10^{-3} M) or the crude preparation of rhodamine bisguanidinobenzoate were used in a similar manner to 9-aminoacridine alone with the minor dif-

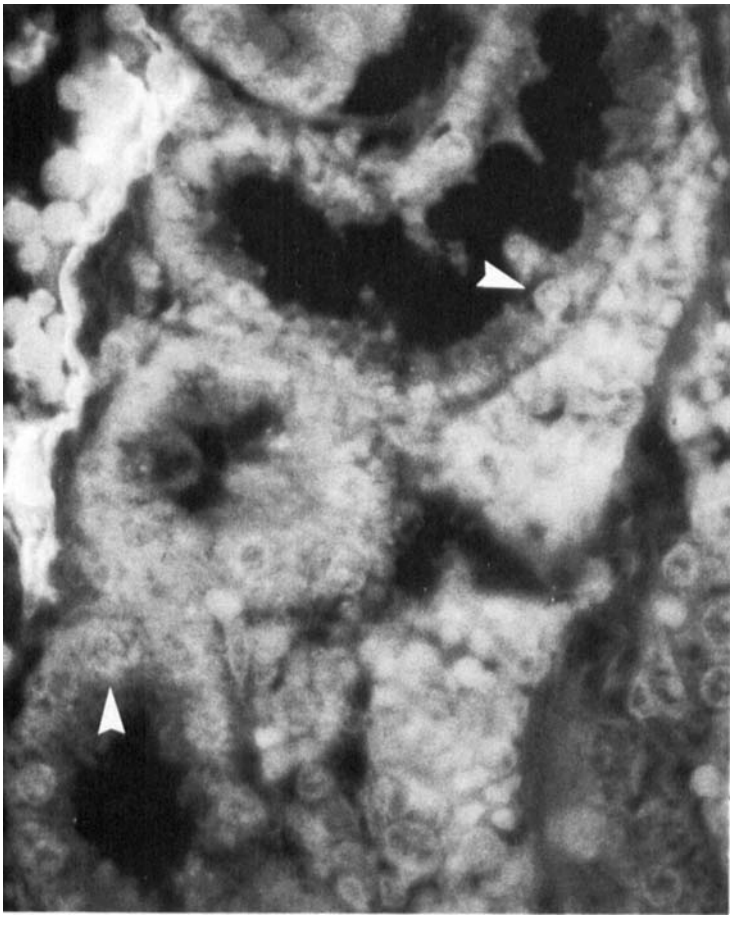

FIGURE 4 Wax sections stained with propidium iodide, magnification × 600. Nuclei of tubule cells are indicated with arrowheads, these nuclei are fully stained and could be confused with similarly sized leukaemia cells in the intertubular spaces. (See Colour Plate IV).

ference that the excess rhodamine bisguanidinobenzoate was washed out of the sections with isotonic saline for 1 h prior to microscopic analysis.

We employed a Leitz orthoplan fluorescent microscope and violet illumination, filming was carried out with Kodak ASA 400 colour film in an Olympus OM-2N camera.

RESULTS AND DISCUSSION

9-Aminoacridine staining of these kidney sections clearly demonstrated the presence of leukaemia cells surrounding tubules and glomeruli. The leukaemia cells exhibited a yellow fluorescence distributed over the whole surface area of the cell. It was also noted that the nuclei of normal kidney tubule cells were faintly ring stained (Figure

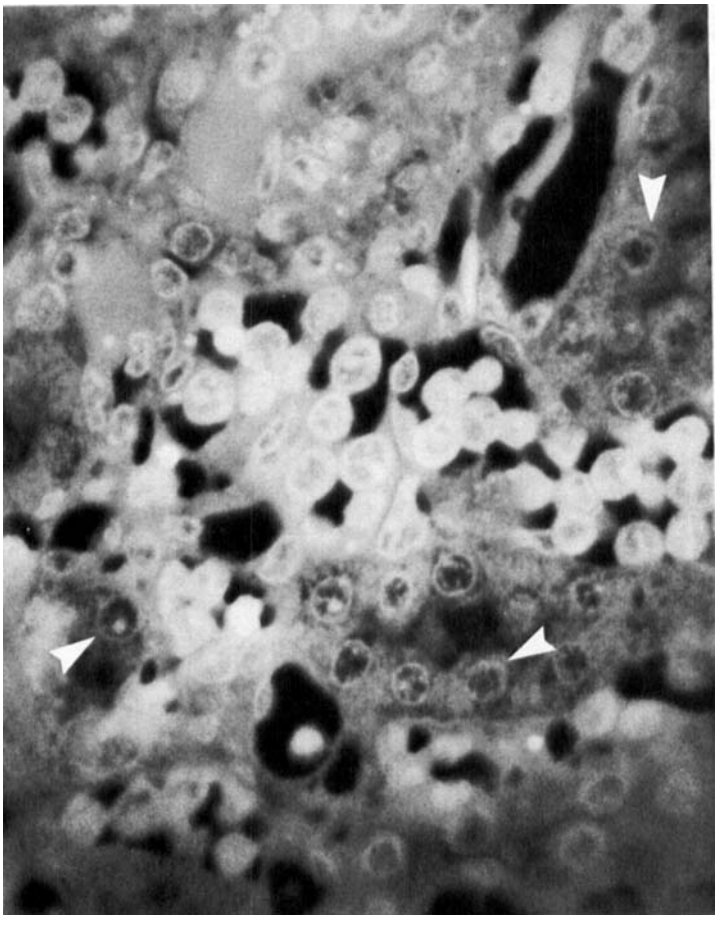

FIGURE 5 Resin section stained with propidium iodide only, magnification × 600. The ring staining of the tubule cell nuclei is particularly obvious (arrows) whilst the leukaemia cells are stained pink over their surfaces. (See Colour Plate V).

1, arrowheads). Double staining of the sections with 9-aminoacridine followed by propidium iodide resulted in pink staining of leukaemia cell surfaces and pink ring staining of normal nuclei (Figure 2). It was noted that the interior of the nuclei of tubule cells did not stain under these conditions with propidium iodide; in contrast to the total staining of nuclei in wax embedded sections (Figure 3). Direct propidium iodide staining of the wax embedded sections (Figue 4) also resulted in total staining of the nuclei of tubule cells as well as the surface staining of the leukaemia cells. The nuclear staining in the wax sections (Figures 3 and 4) should be compared with the perinuclear staining observed in the resin section (Figure 5) stained with propidium iodide alone.

The absence of nuclear staining of resin sections treated with propidium iodide revealed the nuclear membrane staining seen in the cells of the tubules in Figures 2 and 5 and suggested that propidium iodide was binding to the same sites that attract

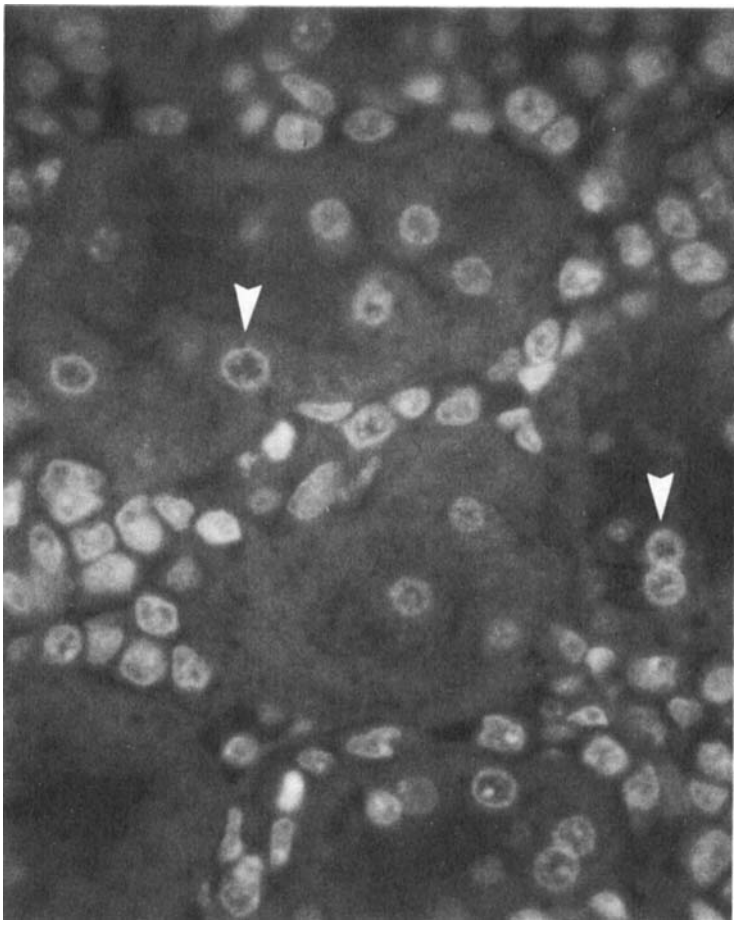

FIGURE 6 Resin sections stained with dansyl sulphaguanidine, magnification × 600. Positive staining is indicated by pale blue fluorescence of the tubule cell perinuclear region (arrows) and the total staining of the leukaemia cells in the intertubular spaces. (See Colour Plate VI).

9-aminoacridine (Figure 1). This evidence suggested that propidium iodide might also be a competitive inhibitor of guanidinobenzoatase. This hypothesis was confirmed by kinetic analysis which showed that propidium iodide was weakly bound to the enzyme with a K_i value of approximately 8×10^{-4} M. This would explain why propidium iodide can be used to locate cells possessing guanidinobenzoatase which have been stained with 6×10^{-5} M propidium iodide in Figure 5, in the absence of a competitive substrate (e.g. 4-methylumbelliferyl-*p*-guanidinobenzoate[1]). The advantage of cell surface staining in the absence of nuclear staining is obvious when studying cells such as leukaemia cells which possess a large nucleus and little cytoplasm. This advantage appears to be confirmed by the resin sections (Figures 1, 2 and 5) as compared to the wax embedded sections (Figures 3 and 4) and frozen sections (not shown).

Dansyl-sulphaguanidine has previously been used to locate tumour cells in a

manner similar to 9-aminoacridine staining[2] in wax embedded sections. In this case, the guanidino moiety directed the fluorescent probe to the active centre of guanidinobenzoatase. When used on resin sections of leukaemic rat kidney tissue (Figure 6), dansyl-sulphaguanidine bound to the surface of tumour cells and also weakly stained the nuclear membrane of the cells in the normal tubules. In this case, the fluorescent probe has no affinity for DNA and consequently would not be expected to cause nuclear fluorescence. These observations confirm the conclusions made with respect to the propidium iodide staining of leukaemia cells and nuclear membranes of host cells. In each case the central region of the host cell nuclei remained unstained in resin sections.

Propidium iodide, 9-aminoacridine and dansyl sulphaguanidine are all competitive inhibitors of guanidinobenzoatase. These reagents bind to the active centre in a reversible manner in the absence of a competitive substrate, which means that the fluorescent probes are unopposed in their approach to the active centre. This concept is the basis of the fluorescent labelling technique employed in tumour cell location[2]. Kinetic studies with soluble guanidinobenzoatase in the presence of 4-methylumbelliferyl-*p*-guanidinobenzoate define which fluorescent compounds are likely to be satisfactory probes for the location of cell-bound guanidinobenzoatase.

The new reagent, rhodamine bisguanidinobenzoate has been shown to be an active site titrant for guanidinobenzoatase[10], one molecule of rhodamine monoguanidinobenzoate being formed for every molecule of enzyme inhibited. We wondered whether the rhodamine monoguanidinobenzoate generated in this inhibition reaction could bind to the active centre of guanidinobenzoatase rather than the guanidinobenzoate which is simultaneously produced in this initial cleavage. Guanidinobenzoate would seem to be an unlikely inhibitor at these low concentrations since it is also formed in the guanidinobenzoatase cleavage of 4-nitrophenyl-*p*-guanidinobenzoate, 4-methylumbelliferyl-*p*-guanidinobenzoate[1] and fluorescein-monoguanidinobenzoate[10]; these are all substrates for this enzyme. The resin sections of leukaemic rat kidney were treated with the crude preparation of rhodamine bisguanidinobenzoate and the slides were washed for 18 h in running water. It was observed that the leukaemia cells stained very clearly, while in the surrounding kidney tubule cells the nucleus was faintly ring stained (Figures 7 and 8). The surface staining of the leukaemia cells and the ring staining of the tubule nuclei were inhibited by prior treatment of the sections with diisopropyl-fluorophosphate, phenylmethyl sulphonyl fluoride[1,2] or meta aminobenzamidine[10], all of which are known inhibitors of serine proteases and, in particular, guanidinobenzoatase. It should be pointed out that rhodamine itself did not stain leukaemia cells although the complex with guanidinobenzoate inhibited guanidinobenzoatase in solution and stained the leukaemia cells. The ability of crude rhodamine bisguanidinobenzoate to inhibit and simultaneously label cells possessing this enzyme may offer a possible method for following the fate of pre-labelled leukaemia cells re-injected into normal rats. The intense fluorescence of these cells should permit their location in unstained sections of host tissue.

Although the work described in this report is confined to kidney tissue, similar results were obtained in liver, testis and epididymis resin sections obtained from leukaemic animals; the leukaemia cells were clearly defined by the fluorescent probes described above.

The binding of these fluorescent probes to this surface protease offers an excellent test system to demonstrate the arrival of other molecules targeted to the same active centre. Two approaches are possible, the simplest is to modify the compound by

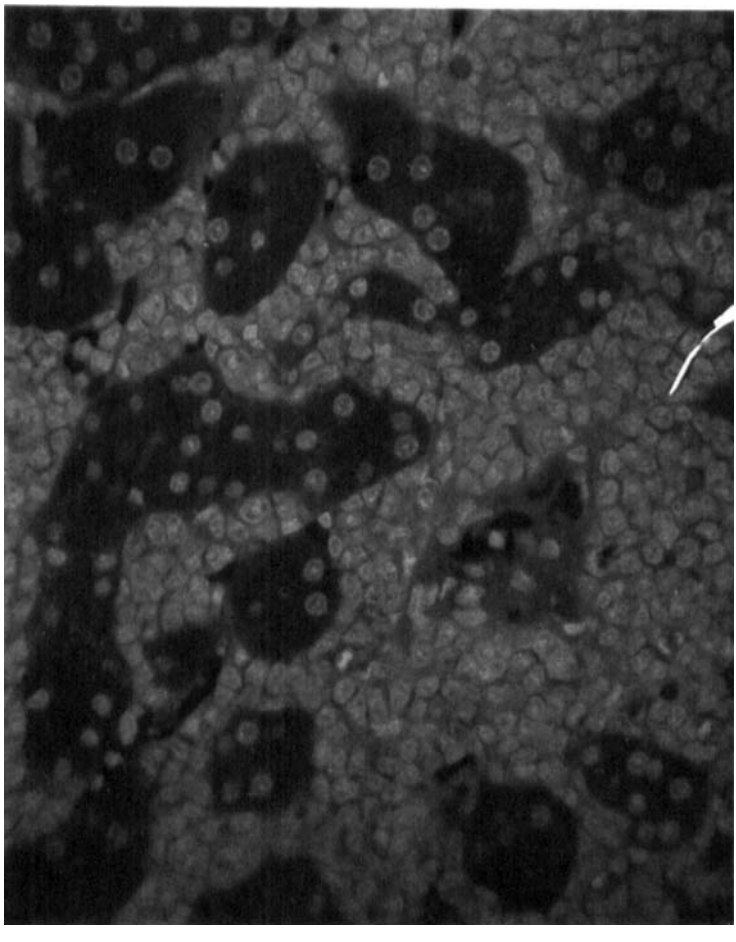

FIGURE 7 Resin section stained with crude rhodamine bisguanidinobenzoate, magnification × 300. Positive staining is indicated by organge-yellow fluorescence. The perinuclear staining of the tubule cells is clearly distinguished from the total staining of the leukaemia cells. The leukaemia cells are packed into the intertubular spaces. (See Colour Plate VII).

including a fluorescent moiety, for example the drug, sulphaguanidine can be dansylated and employed as shown in Figure 6. Alternatively, a non-fluorescent but liganded molecule (such as sulphaguanidine itself) can be used to compete with 9-aminoacridine for the binding site on the cell surface; in this case the 9-aminoacridine staining can be abolished or greatly diminished. It should be possible to target cytotoxic drugs with suitable ligands for guanidinobenzoatase and attract these drugs to tumour cells. This technique can provide information on the arrival of a desired molecule at the surface of leukaemia cells but gives no information as to whether the molecule is taken into the tumour cells and metabolised *in vivo*.

In conclusion, we have employed three competitive and one non-competitive inhibitor of guanidinobenzoatase to leukaemic kidney tissue sectioned in resin. In each case the inhibitor carried a fluorescent moiety which enabled the cells possessing

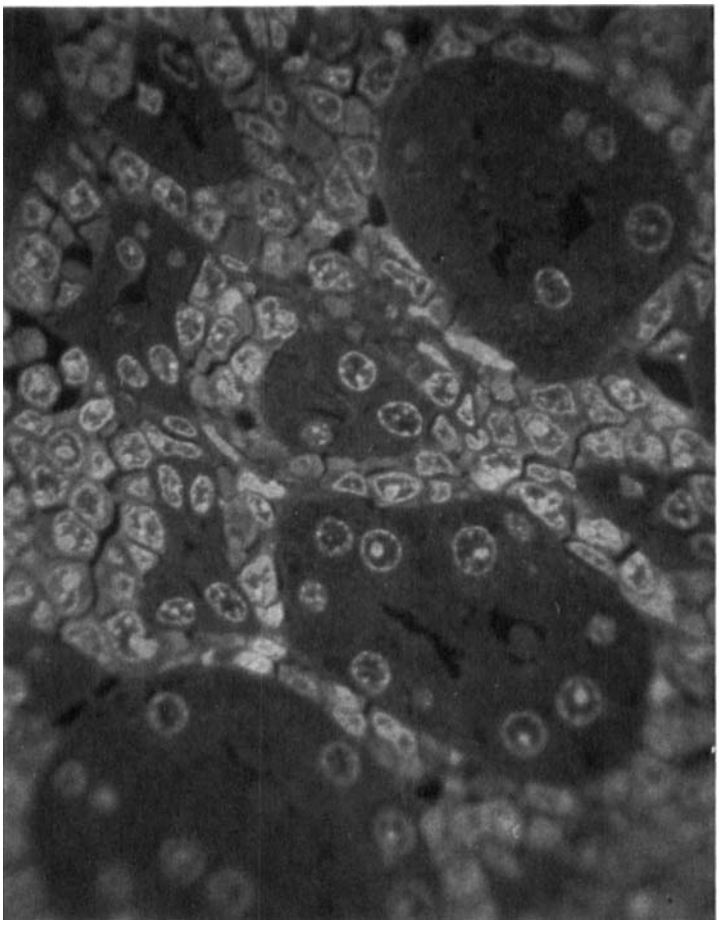

FIGURE 8 Resin section stained with crude rhodamine bisguanidinobenzoate, magnifications × 600. The orange-yellow fluorescence of the leukaemia cells and the perinuclear regions of the tubule cells can be clearly identified. (See Colour Plate VIII).

this enzyme to be located by their surface fluorescence. The resin sections have two advantages over wax embedded sections: (a) the nuclei do not bind these probes, and (b) the tissue structure is not shrunken and is a closer approximation to the natural state. The binding of fluorescent probes to cells possessing guanidinobenzoatase can be anticipated by a study of the inhibition kinetics of guanidinobenzoatase in solution[1]. It would also seem that the weak competitive inhibitor propidium iodide can be used to locate leukaemia cells in resin sections without the need to employ double staining and stacking of propidium iodide on 9-aminoacridine molecules at the active centre of the enzyme. Since propidium iodide provides excellent contrast and the staining technique is so simple, it would seem that this might be preferred for locating leukaemia cells in kidney resin sections. These inhibitors have been used as though they were fluorescent antibodies for guanidinobenzoatase and have shown this enzyme to be located on the surface of leukaemia cells as well as in the perinuclear

region of host tubule cells. Clearly this technique could be used with other enzymes and suitable low molecular weight fluorescent inhibitors for these enzymes, the selectivity being confirmed by inhibition kinetic studies of the enzyme in true solution[10].

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References

1. F.S. Steven and R.K. Al-Ahmad, *Eur. J. Biochem.* **130,** 335–339 (1983).
2. F.S. Steven, M.M. Griffin and R.K. Al-Ahmad, *Eur. J. Biochem.* **149,** 35–40 (1985).
3. P.L. Coleman, H.G. Latham and E.N. Shaw, *Meth. Enzymol.* **45,** 12–26 (1976).
4. F.S. Steven, M.M. Griffin, T.L.H. Wong, B.S. Brown and I.M. Jeffrey, *Biochem. Soc. Trans.* (in press).
5. F.S. Steven, M.M. Griffin and R.K. Al-Ahmad, *J. Chromat.-(Biomed. Application)* (in press).
6. W.R. Gray and B.S. Hartley, *Biochem. J.* **89,** 379–380 (1963).
7. S.P. Leytus, W.L. Patterson and W.F. Mangel, *Biochem. J.* **215,** 253–260 (1983).
8. M. Didley, S. Dorsch and B. Roser, *Pathology* **7,** 219–235 (1975).
9. H. Jackson, N.C. Jackson, M. Bock and M. Lendon, *Br. J. Cancer* **50,** 617–624 (1984).
10. F.S. Steven, M.M. Griffin, T.L.H. Wong, R. Yasmin and W.F. Mangel, *J. Enz. Inhib.* **1,** 187–201 (1987).