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LOCATION OF THE TUMOUR CELLS IN THE SPUTUM SPREADS WITH FLUORESCENT PROBE PENTOSAN POLYSULPHATE

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ABSTRACT: Pentosan polysulphate (PPS) is highly negatively charged polysaccharide compound and is significant inhibitors of Guanidinobenzoatase (GB), associated with tumour cells in the sputum spreads of lung cancer patients, and free GB in solution. Texas red labeled PPS (TR-PPS) selectively recognized and binds the GB associated with the tumour cell surfaces, and these cells fluoresced slightly black-red. 9-aminoacridine (9-AA) is another low molecular weight yellow fluorescent inhibitor of GB and can recognize and bind with both isozymic forms of GB, associated with tumour and normal tissues. If the sputum spreads are stained with 9-AA, they behave similarly as with TR-PPS in recognizing and binding with this probe and fluoresce brightly yellow under the fluorescent microscope. Kinetic studies have shown that PPS inhibit the fee GB in solution in a concentration-dependent manner. These studies suggest that TR-PPS is a potent inhibitor of tumour cells GB and can be used as a novel fluorescent probe for the location of tumour cells in the sputum spreads of the patients suffering from lung cancers.

Key words: Tumour protease, proteases inhibitors, sputum spreads, Pentosan polysulphate, Guanidinobenzoatase, fluorescence microscopy.

INTRODUCTION

Regulation of the extracellular matrix by proteases and protease inhibitors is a fundamental biological process for normal growth, development and repair (Korolenko *et al.,* 2008; Koistinen *et al.,* 2008; Wang *et al.,* 2008). Matrix metalloproteinases, and the serine proteases are the major extracellular-degrading enzymes involved in

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extracellular degrading enzymes involved in extracellular matrix degradation (Wilson *et al.,* 2008). Normally, the highly integrated action of these enzyme families remodels all of the components of the matrix and performs essential functions at the cells surface involved in signaling, cell survival, and cell death. During the inflammatory response induced in infection, autoimmune reactions hypoxia, abnormal expression and activation of these proteases lead to breakdown of the extracellular matrix which result in the opening of the blood-brain barrier, prevention of normal cell signaling, and eventually leading to cell death (Candelario-Jalil *et al.,* 2008). Proteases are more sensitive indicators and have a major prognostic impact for colorectal cancer than the commonly used tumour markers at the time of clinical detection (Herszenyi *et al.,* 2008).

Tumour cells and cells capable of migration possess a protease Guadanidinobenzoatase (GB), which is similar in function to the tissue plasminogen activator but not identical with it (Steven *et al.,* 1991 and Poustis *et al.,* 1992). GB may exist in different iso-enzymic forms and can be located by different fluorescent inhibitors both in cryostat and wax embedded sections under fluorescent microscope (Steven, *et al.,* 1991). 9-AA is an active site directed low molecular weight, competitive inhibitor of GB and can be used for the location of both isozymic forms of GB, which after binding make the cell surfaces sluoresced yellow (Steven *et al.,* 1985; Steven *et al.,* 1994; and Murza *et al.,* 2000). PPS is a semisynthetic, polysulphated sacchride, which have been previously investigated investigated as an anti-coagulant, anti-angiogensesis, anti-haemorrhagic cystitis and anti-metastatic agent (Nguyen *et al.,* 2000 Yunmbam *et al.,* 1998; Sandhu *et al.,* 2004; Rha *et al.,* 1997; Mcleskey *et al.,* 1996). Tumor angiogenesis is critically important to tumor growth and metastasis and it have shown that PPS is an effective inhibitor of heparin-binding growth factors in vitro ad can effectively inhibit the establishment and growth of tumors in nude mice (Marshal *et al.,* 1997). The particular interest shown in this compound was that it could be used as a non-toxic compound to inhibit the growth of prostate cancer at an early stage, when the tumour is not clinically evident (Zaslu *et al.,* 2004). PPS is the only Food and Drug Administration-approved oral therapy for cystitis and may also have a therapeutic effort on breast cancer cells in vitro (Zaslau *et al.,* 2004). Therapeutic potential was observed by the administration of PPS, which immensely decreased proliferation as well as extracellular matrix

production in osteoarthritis and prostate smooth muscles (Elliot *et al.,* 2003). In present studies interactions of PPS have studied by GB bound with membranes in sputum spreads and free GB in solution, by employing the conventional kinetic analysis. TR-PPS selectively recognize and bind the GB associated with the tumour cells surfaces and these cells fluoresced red under the fluorescent microscope. However, TR-PSS fails to recognize and bind the surrounding normal epithelial cells surfaces in the sputum spreads; hence these cells lacked red fluorescence and appeared as slightly black-red. From these studies it is concluded that PPS is potent inhibitor for the carcinoma tissues GB and could be of value in diagnostic pathology and possibly in therapeutic medicine by coupling with cytotoxic drugs or radioactive metals for selective killing of tumour cells.

MATERIALS AND METHODS

Lung carcinoma tissues were used for the extraction GB (Murza *et al.,* 2000 and Anees, 1996) and 48 sputum spreads of the lung cancer patients were provided by Dr. I. C Talbot, St. Marks Hospital, London. PD-10 disposable columns were purchased form Pharmacia/LKB, Uppsalla, Sweden 9-AA, Texas red acid chloride, cynogens bromide and pentosan poysulphate were purchased from sigma chemical Co. Ltd, St. Louis, USA. *Coupling of PPS with Texas Red Acid Chloride* Texas red acid chloride (0.5 mg/ml) was first converted into Texas red amine, by condensation of the reactive chloride with ammonia solution, and the excess ammonia was removed by evaporation according to the previous studies (Anees, 1996). Texas red amine was coupled to PPS by cyanogens bromide activation. In brief, 1 ml of cynogens bromide (5mg/ml) was added to 1 ml of PPS (5mg/ml) at pH 10, and the reaction was allowed to proceed for 3-4 minutes. Texas red amine (5ug) was added and the reaction mixture was left overnight with constant shaking the reaction products were passed over a PD-10 column for the separation of free Texas red amine from the red labeled TR-PPS, which has a higher molecular weight.

TR-PPS/9-AA Staining

The staining was carried out by covering each spread on the slide with 10µl of TR-PPS (9×10^{-5}M) and 9-AA (1×10^{-4} M) for 30 minutes, followed by washing off the excess stain in fresh isotonic saline for 1minute (Steven *et al.,* 1992). Dissociation of Enzyme-inhibitor complex with 2% SDS solution was used to remove the inhibitor from the active site of the GB as described by Steven *et al.,* (1992). Assay of GB by fluorogenic substrate4-methylumbelliferyl-guanidinobenzoate (MUGB) GB was purified from lung carcinoma tissues as described earlier by using agmatine-sepharose affinity chromatography (Murza *et al.,* 2000 and Anees, 1996). The GB activity was assayed with MUGB as a substrate; the fluorescent product 4-methyllumferone (MU) was measured by fluorescence spectrophotometer. Cleavage of the substrate was monitored at an excitation wavelength of 323 nm and an emission wavelength of 446 nm (Anees, 1996).

Kinetic of Inhibition of GB

Inhibition experiments (Steven *et al.,* 1993) were carried out by pre-incubating purified GB (10µg/ml) with the inhibitor for 10 minutes at 370 prior to adding the substrate ((1×10^{-4} M final concentration).

Fluorescence Microscopy and Photography

Sections stained with TR-PPS were examined in the Leitz Diaplan fluorescence microscope, fitted with an automatic camera and Kodak. ASA 400 colour film was used to record the data (Steven *et al.,* 1992). Under these condition cells with active GB were recognized by TR-PPS exhibited red cell surface fluorescence.

RESULTS AND DISCUSSION

TR-PPS binds GB associated with the tumour tissues in the sputum spreads and the cell surfaces after binding with TR-PPS fluoresced bright red under the fluorescent microscope. Whilst, the surrounding normal epithelial cells which lack GB could not bind with TR-PPS and do not fluoresced red. When these TR-PPS treated sections were challenged with another yellow fluorescent probe 9-AA, the active center of GB on these tumour cells surfaces failed to recognize and bind 9-AA and could not

fluoresce yellow. However, there was a little binding of 9-AA, due to its co-stacking with the TR-PPS, and the cell surfaces fluoresced as yellowish-red. When these TR-PPS/9AA stained sputum spreads were treated with SDS for five minutes and then re-challenged with 9-AA, the tumour cells bind this probe and showed yellow fluorescence. These results indicate that active centers of GB on cell surfaces were already blocked with TR-PPS and 9-AA could bind with these cells and they do not fluoresced yellow. After the treatment with SDS, the inhibitor TR-PPS was removed and 9-AA bind with GB and showed yellow fluorescence.

These findings were in a agreement with the previous studies that TR-PPS selectively recognize and bind the active GB associated with tumour cells and fail to recognize and bind GB when it is present as enzyme-inhibitor complex (Anees, 1996) or the cell surfaces lack GB (normal epithelial cells of lung tissues) and do not fluoresce red/yellow under the fluorescent microscope.

Another evidence for the binding of GB with TR-PPS comes from the kinetic studies which have confirmed the above findings, when free GB was studies in solution, instead of bound to the intact membranes. GB continuously hydrolyzed MUGB with the production of fluorescent product MU. When the inhibitor PPS was added into the medium it inhibited the GB activity, with the increase of inhibitor concentration. A maximum inhibition was achieved at the concentration of 9×10^{-5} M. These results were similar to the previous findings (Steven and Talbot 1994, Anees, 1996), which further support that both 9-AA and TR-PPS bind the tumour cells.

From the above results it is concluded that TR-PPS selectively bind to the tumour cells in the sputum spreads and fail to recognize the normal epithelial cells which lacked GB. Therefore, TR-PPS could be used diagnostically for the location of tumour cells in the sputum spreads, Since, PPS is a nontoxic agent and it could be used in the future as a carrier of cytotoxic drugs for the selective destruction of tumour cells with in the lung tissues.

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