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FOREWORD

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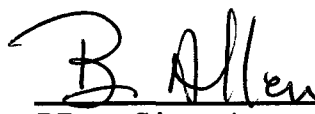
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INTRODUCTION

The major failure in breast cancer management is due to incomplete killing of malignant tumour cells that have metastasized (Allen 1999a). A novel approach that may overcome this problem in breast cancer management is named "Targeted Alpha Therapy" (TAT) using the components of plasminogen activation system that is involved in the metastatic spread of breast cancer cells. Targeted alpha therapy uses an alpha emitting radionuclide as a lethal medicament via an effective targeting carrier to cancer cells for the purpose of cancer control (Allen 1999b). In this case the targeting carrier is the plasminogen activator inhibitor type-2 (PAI2) which specifically targets the urokinase plasminogen activator (uPA) that is over-expressed on malignant cells. Biotech Australia supplies recombinant human PAI2 under a material transfer agreement. We now report the first chelate of the alpha emitting radioisotope ^{213}Bi to PAI2 via cDTPAa, and its stability, specificity, in vitro cytotoxicity and in vivo control for two human breast cancer cell lines that differ in metastatic phenotypes MDA-MB-231 and MCF-7 (Ranson et al. 1998).

BODY

Task 1 Establish radio-nuclide supply

The Ac-Bi generator is purchased from the USDOE on a regular basis. Elution conditions for the extraction of Bi-213 have been optimized using 0.15 M distilled and stabilized hydriodic acid. We can milk the generator every two hours as required. The laboratory has been upgraded for high activity alpha radiation with the installation of seamless work trays and lead glass window shields.

Task 2 In vitro testing

We have not yet attempted to chelate Bi-213 with the uPA antibody because of the success experienced so far with the alpha-PAI2 approach (see below). The Tc-99m and I-131 labeling projects for determining diagnostic sensitivity in solid tumours has not yet been done because of the success of the alpha-PAI2 work.

A Labeling of PAI2.

PAI2 (1 mg) dissolved in PBS was conjugated to cDTPAa by first increasing the pH to approximately 8.2 via the addition of 10% (v/v) 1 M NaHCO_3 (pH 9.0). A 50 fold molar excess of cDTPAa was added and the reaction mixture incubated at 25°C for 1 h with intermittent rocking. Three reaction volumes of PBS were used to purify DTPA-labeled PAI2 away from free cDTPAa using a microspin concentrator.

Concentrated DTPA-PAI2 stock was diluted as appropriate with 500 mM sodium acetate at pH 5.5 and labeled with free ^{213}Bi for 20 minutes at room temperature. After labeling, ^{213}Bi -DTPA-PAI2 was buffer exchanged into PBS using a PD-10 column using PBS (pH 7.0) as the eluting buffer.

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We have achieved labeling efficiencies for Bi-213 of up to 95% with both cDTPAa chelators, produced stable alpha-PAI2 with ~20% leaching after 2 half lives in serum. The inhibitory activity of alpha-PAI2 has been confirmed by its ability to form complexes after 40 min incubation at 20°C with active uPA. Complex formation was detected by a molecular mass shift by SDS-PAGE. The stoichiometry of chelate to PAI2 is currently being investigated and the protocol is being worked up in collaboration with protein chemists at Biotech Australia.

B In vitro efficacy

The specific cytotoxicity of alpha-PAI2 in vitro has been established. Table 1 shows cell survival data based on 37% survival dose (Do) for two cell lines (MDA-MB-231 and MCF-7, high and low levels of cell-surface uPA antigen, respectively). Results show that:

- The specific uPA activity blocking agent [EGR-chloromethylketone (EGR-CMK), this effectively inhibits PAI2 binding (Hang et al 1998)] significantly improves survival by a factor of 2.5 as a result of inhibition of the PAI2 interaction with cellular uPA. Non-specific alpha-BSA had little cytotoxic effect.
- MDA-MB-231 and MCF-7 have similar survivals (ie ~ 2 µCi for 37%). This implies that since only a few alpha hits of the nucleus are required to kill a cell, compared with thousands for betas, even tumour cells with low uPA levels (ie MCF-7) can still receive a lethal dose.
- No cytotoxicity was observed with freshly isolated normal human leukocytes reflecting that non-targeted cells are immune from alpha-PAI2.

Cells	Alpha-protein	µCi		% Survival
		- EGR-CMK	+ EGR-CMK	
MDA-MB-231	Alpha-PAI2	2.0 ± 0.2	5.3 ± 0.3	37
	Alpha-BSA	4.9 ± 0.1	-	87
MCF-7	Alpha-PAI2	2.3 ± 0.4	4.9	37
	Alpha-BSA	4.8	-	84
Fresh leukocytes	Alpha-PAI2	5.0	-	100
	Alpha-BSA	5.0	-	89

We have also shown by confocal microscopy that uPA/PAI2 complex is specifically internalised (presumably by endocytosis) by the MDA-MB-231 cells. Internalisation improves the cytotoxicity for isolated cells as the probability for alpha tracks crossing the nucleus is increased.

Task 3 In vivo studies

We have established a breast cancer xenograft model that mimics the early human breast cancer metastatic pathway that is via lymphatic drainage around the breast. Briefly, xenografts of human metastatic breast cancer cell line MDA-MB-231 are induced by sc inoculation of 2×10^6 cells /site into first pair mammary fat

pads on the thoracic wall of 4-6 week old female athymic nude mice. To date, visible tumours were observed in 27/30 sites inoculated after 2 weeks. After 4 weeks macroscopic signs of angiogenesis are obvious around the tumour and surrounding skin. Dissection and histological examination of tissues at this stage confirmed the presence of human tumour cells in the mammary fat tissue (ie primary tumour) as well as in the axillary and cervical lymph nodes. We have data indicating the presence of uPA antigen in both the primary tumours and metastatic cells in these regional lymph nodes. Thus such a model will be useful for further TAT studies mimicking patients presenting with early stage breast cancer who have already undergone local therapy. This model will be used to study the inhibition of metastases by local and systemic TAT. Under anaesthesia, the primary tumour in the mammary pad will be removed, and TAT given at various post-surgery times sc or iv routes. The optimum dose (based on toxicity studies) and time will be determined to control the growth of tumours in the lymph nodes. Control animals will receive PBS or alpha-BSA.

A post-inoculation efficacy study will also be made for *systemic* TAT for each model. Tail vein (tv) injections will be made using a restraint or light inhalation anaesthesia combined with the use of Temgesic and/or the application of EMLA cream prior to the procedure. The API tail vein injections will be given to the respective groups at the optimum post-inoculation time found for local TAT. A comparison of the results from different four groups of mice will clearly indicate the efficacy of TAT. In addition, the inhibitory effect of the API on 2-5 mm lesions will be investigated.

Our toxicology studies show that nude mice can tolerate up to 3 mCi/kg of alpha-PAI2 by systemic administration, causing only up to 10% temporary weight loss compared to the chelated-PAI2, PAI2 and PBS control mice. These results are consistent with the toxicity of Bi-213-labeled monoclonal antibodies reported by Scheinberg et al at the Memorial Sloan Kettering Cancer Institute. Doses up to 1 mCi/kg of Bi-213-MAb have been administered to recurrent acute myelogenous leukaemia patients without serious complications, although myelosuppression has been observed in a few patients. Local TAT can completely inhibit the growth breast cancer xenografts in nude mice with only 25 μ Ci injection (Fig 1).

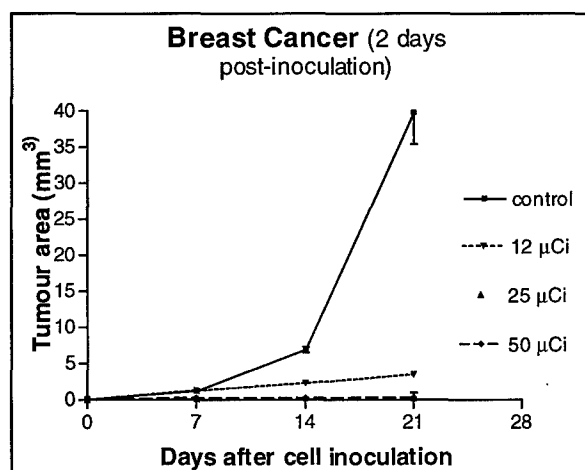


Fig 1 Inhibition of breast cancer by alpha-PAI2, showing tumour area vs post-inoculation time for control (PBS), 12.5 μ Ci and complete inhibition for 25 and 50 μ Ci alpha-PAI2 administered sc 2 days post inoculation. 2×10^6 MDA-MB-231 cells were inoculated sc into the mammary fat pad on both sides of 6 week nude mice.

Thus the preclinical and early clinical data point to the fact that alpha-PAI2 may well be an efficacious and safe therapeutic modality and that TAT is therefore expected to solve the problems of variable antigenic expression, isolated or small cell cluster toxicity, preangiogenic lesions as blood flow would quickly carry the alpha-PAI2 to microscopic lesions.

KEY RESEARCH ACCOMPLISHMENTS

- 1 Ac-Bi generator supply and elution established.
- 2 Stable and active chelated ^{213}Bi -PAI2 (alpha-PAI2) has been synthesised for the first time.
- 3 Excellent in vitro specific cytotoxicity of breast cancer cells has been demonstrated for the first time with alpha-PAI2.
- 4 In vivo toxicity measurements show that therapeutic doses of alpha PAI2 can be delivered to mice without serious complications.
- 5 Complete inhibition of the growth of breast cancer tumours has been achieved by local alpha-PAI2 injection at 2 days post-inoculation.

REPORTABLE OUTCOMES

Manuscript submitted:

Reviews in Hematology and Oncology (attached).

Allen et al: "In vitro and preclinical targeted alpha therapy for melanoma, breast, prostate and colorectal cancers".

Manuscript in preparation:

Z Tian, B J Allen, N M Andronicos, S Rizvi, M Ranson: "Targeted alpha therapy for breast cancer via plasminogen activation system".

Patent applied for and/or issued:

Australian provisional patent number PQ5824, file date 24/2/2000, expiry date 24/2/2001 "A method of treatment and agents therein for cancer". Filed in the name of Biotech Australia Pty Ltd, University of Wollongong, Medical Scitec Australia Pty Ltd (attached)

Funding applied for based on work supported by this award:

NHMRC (Australia) Development Grant Application for 2001-3: "Targeted alpha therapy: development of a new treatment for metastatic breast cancer."

Principle Investigators: M Ranson (University of Wollongong), B J Allen (St George Hospital & University of Wollongong), C L Bunn (Biotech Australia).

NHMRC (Australia) Project Grant Application for 2001-3: "Preclinical trials of multi-targeted alpha therapy for prostate cancer". B J Allen (St George Hospital), P J Russell (Prince of Wales Hospital), M Ranson (University of Wollongong).

USDOD Grant Application for 2001-3: "Targeted alpha therapy with labeled monoclonal antibodies, protein and octopeptide for the control of micrometastatic prostate cancer". B J Allen (St George Hospital).

CONCLUSIONS

The research so far supports the use of a new targeting protein, PAI2, to target breast cancers cells and the alpha emitter Bi-213 to kill these targeted cells. As such, we are clearly moving towards the development of a new therapeutic modality for breast cancer. Under the material transfer agreement for PAI2, Biotech Australia has the first option for use of alpha-PAI2 for commercial development. They are currently funding optimisation studies for GMP production of PAI2 for clinical use.

So what?

If ultimately successful, alpha-PAI2 therapy can change the prognosis for many breast cancer patients who are clinically free of disease but who have a high probability of micrometastatic disease which would eventually lead to a reduced life span. We are clearly well on the way to establishing the basic preclinical conditions required before we move to the clinical trial stage.

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Hang MTH, Ranson M, Saunders DN, Liang X-M, Bunn CL, and Baker MS. (1998) Pharmacokinetics and biodistribution of recombinant human plasminogen activator inhibitor type 2 (PAI-2) in control and tumour xenograft-bearing mice. Fibrinolysis and Proteolysis. 12:145-154.

Ranson M, Andronicos NM, O'Mullane M. and Baker MS. (1998) Increased plasminogen binding is associated with metastatic breast cancer cells: Differential expression of plasminogen binding proteins. British J. Cancer. 77: 1586-1597.

APPENDICES

- 1 Paper by Allen et al. for Hematology and Oncology.
- 2 Provisional patent application.

July 18, 2000

**IN VITRO AND PRECLINICAL TARGETED ALPHA THERAPY FOR
MELANOMA, BREAST, PROSTATE AND COLORECTAL CANCERS**

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Abstract



Targeted alpha therapy (TAT) offers the potential to inhibit the growth of micrometastases by selectively killing isolated and preangiogenic clusters of cancer cells. The alpha emitting radioisotopes Tb-149 and Bi-213 were produced by accelerator and generator, respectively, and chelated to cancer affined monoclonal antibody, peptide or protein to form the alpha-immunoconjugates (AIC) and alpha-plasminogen activation inhibitor type-2 (API) against melanoma, leukaemia, breast, prostate and colorectal cancers.

These conjugates are tested for stability, specificity and cytotoxicity, and found to be highly specific and cytotoxic in vitro. Subcutaneous inoculation of one million melanoma or breast cancer cells into the flanks or mammary pads, respectively, of nude mice causes tumours to grow in all mice. Melanoma and breast cancer tumour growth is obtained for untreated controls, nonspecific and specific TAT, for 2 days post-inoculation subcutaneous injection models. Only for TAT mice is complete inhibition of melanoma growth found. Intra-lesional TAT is very successful in melanoma, with all melanomas being regressed for 100 μ Ci injections. However, only partial responses were observed for intra-lesional TAT of breast cancer tumours.

These results point to the potential application of local TAT in the management of recurrent sc cancer and invasion of lymph nodes.

1. Introduction

The control of cancer continues to be an elusive objective. While current therapies can be effective, in general they fail to change prognosis in the more common cancers.

Melanoma has its highest incidence in Australia, and while surgery for early stage melanoma can be curative, there is no systemic therapy available to control metastatic melanoma.

Colorectal cancer (CRC) is lethal if the cancer has spread beyond the colonic wall. Systemic therapies achieve a 20-40% objective remission rate with only a marginal improvement in survival [1,2]. Currently ^{131}I -Lipiodal therapy can partially regress CRC liver metastases but does not lead to cure [3,4].

Prostate Cancer: Each year some 185000 new cases are reported in the USA, with 39000 deaths [5]. Radical prostatectomy or external beam radiation gives the best chance of a cure (> 10 years) [6], but distressing side-effects like incontinence, impotence, bowel damage and stricture can occur [7].

Breast Cancer: The systemic treatment of early breast cancer by hormonal, cytotoxic or immunotherapy has been exhaustively studied by the Early Breast Cancer Trialists' Collaborative Group [8]. Highly significant reductions in recurrence and death were found for Tamoxifen (25%, 17% resp), ovarian ablation for age<50 y (26%, 25%), and polychemotherapy (28%, 16%) but not for ablation at age > 50 y nor immunotherapy. There were 24 trials of immunotherapy, for which results were particularly disappointing. While current therapies have value, the majority of patients with metastatic cancer did not receive a benefit.

Clearly new therapeutic approaches are urgently needed for all these cancers. Targeted Alpha Therapy (TAT) is such an approach, as it has the potential to deliver a highly

cytotoxic radiation dose to targeted cancer cells, while sparing distant normal cells. In this paper we report progress in the synthesis and testing of alpha-immunoconjugates (AIC) and alpha-plasminogen activator inhibitor (API), using the alpha-emitting radionuclides ^{149}Tb and ^{213}Bi chelated to the targeting carriers. The first cytotoxicity results for Tb-149 AIC are also reported for colorectal cancer.

1.1 Targeting Cancer Cells

The major problem with the management of most cancers is an inability to inhibit the development of metastases that eventually result in death of the patient. Melanoma, colorectal and breast cancer are all curative on early stage presentation. Thus an early systemic therapy could be effective in preventing the development of metastases if targeting of isolated cells, cell clusters and preangiogenic lesions could be achieved. Prostate cancer is a very slow growing cancer, and early dissemination of cancer cells occurs before the disease becomes clinical. Further, because of the significant complications arising from the treatment of primary prostate cancer, improved therapies are needed which will give the patient a better quality of life.

In targeted alpha therapy (TAT), a carrier that selectively targets cancer cells delivers a lethal payload in the form of short range, highly cytotoxic alpha radiation. Two targeting approaches are being investigated in this paper:

- a) monoclonal antibodies (mab) against melanoma, colorectal cancer and prostate cancer,
- b) plasminogen activation inhibitor (PAI-2) to target the urokinase plasminogen activator (uPA) for breast and prostate cancers.

The alpha emitters Bismuth-213 and Tb-149 are chelated to these molecules to form alpha emitting alpha-immunoconjugates (AIC) and alpha-PAI-2 (API).

1.2 Targeted carriers

1.2.1 Monoclonal Antibodies (mab)

The present study uses the monoclonal antibody 9.2.27 that is directed against an antigen expressed on most melanoma (9.2.27) cell surfaces [9]. The c30.6, (chimeric version of the mouse antibody) and 35A7 are expressed against antigens on CRC cell surfaces. The mab J591 targets an external domain of the prostate specific membrane antigen (PSMA) [10]. PSMA expression is seen in all prostate cancers and in most metastatic lesions [10,11,12].

1.2.2 Plasminogen Activation Inhibitor (PAI-2)

The plasminogen activator inhibitor type-2 (PAI-2) targets the urokinase plasminogen activator (uPA). Since uPA has high affinity and specificity for cell-surface localized receptors (ie, uPAR), where it can be inhibited by PAI-2 [13,14], PAI-2 is more likely involved in pericellular proteolysis in which uPA-mediated proteolysis plays an important role, such as tumour cell invasion and metastasis. We have recently established the pharmacokinetics and biodistribution of exogenously administered human recombinant radioiodinated ^{125}I -PAI-2 in both control and tumour-bearing animals [14]. Radio-labeled PAI-2 accumulated in tumour xenografts (~1.5% of total injected dose) without an accompanying increase in major organ toxicity, and that tumour associated PAI-2 correlated with tumour mass. Thus exogenously administered PAI-2 targets uPA expressing tumours cells, particularly those that have or are likely to metastasise. Targeting uPA-overexpressing cells by its natural inhibitor remains an unexploited mode

of attack for prostate cancer malignancy. PAI-2 is a human protein that cannot induce human antimouse antibody (HAMA) response.

1.3 Targeted Alpha Therapy

1.31 Biological Effect

Alpha emitting radionuclides [15] emit alpha particles with energies up to an order of magnitude greater than most of the betas rays, yet their ranges are two orders of magnitude less as alpha particles have a linear energy transfer (LET) which is about ~100 times greater. This is manifested by a high relative biological effectiveness (RBE). As a result, a much greater fraction of the total energy is deposited in cells with alphas and very few nuclear hits are required to kill a cell. Consequently, 100 fold enhancement in radiation dose [16,17] would be delivered to the nucleus of a cancer cell if a "smart" carrier is employed to take the alpha-radionuclide to that cancer cell.

Availability of the alpha nuclides has been the major problem in the past for their large scale scientific and clinical application. Studies have been carried out on ^{211}At and ^{212}Bi [for example 18,19,20,21] with encouraging results, Allen and Blagojevic [15] noted that ^{149}Tb could be a preferred radiolabel with its superior nuclear decay parameters, and Allen et al [22] have reported its production. Bi-213 can be obtained from the Ac-225 generator and the Memorial Sloan Kettering Cancer Centre has pioneered the use of this isotope in the first phase 1 clinical trial for advanced leukaemia [23]. McDevitt et al [24] and Allen [25] have recently reviewed these and other data that point to the potential efficacy of alpha-immunotherapy for subclinical and clinical disease.

1.3.2 Requirements for control of metastases

A therapeutic modality must be able to control the growth of metastatic cancer by killing isolated cells and non-targeted cancer cells, while sparing dose limiting stem cells [26]. Only alphas can kill isolated cells at tolerable dose limits, whereas the low LET of betas makes this a very difficult task within human dose tolerance limits. Alphas have ranges of several cell diameters and can easily traverse the nucleus from any position in the cell, causing double strand breaks in DNA that are difficult to repair. Further, the high cytotoxicity of alphas means that targeted cells can be killed even for quite low levels of epitopic expression; some hundreds or thousand times lower than for betas for the same endpoint, reducing the effect of variable epitope expression. Alphas can reach through several cell diameters, killing any cell in their path with only a few traverses of the nucleus, eg contiguous non-targeted cancer cells, but can also spare distant stem cells because of their limited range of effect.

2. Method

2.1 Radioisotopes

The alpha emitting radioisotopes Tb-149,152 [22] and Bi-213 are produced by accelerator and generator respectively and are chelated to a cancer affined monoclonal antibody or protein to form the alpha-immunoconjugate (AIC) against melanoma, leukaemia, and colorectal cancers, and alpha-PAI2 (API) against prostate and breast cancers. These conjugates are tested for stability, specificity and cytotoxicity.

2.2 Chelates

Two chelates are used to couple the radioisotope to the carrier, viz cyclic anhydride of DTPA (cDTPAa) and CHX-A''[27]. Both are known to form stable complexes with monoclonal antibodies. The chelation methods have been described by Rizvi et al [28].

2.3 Cell Lines

MM138: A non-pigmented melanoma cell line supplied by Dr A Henniker, Westmead Hospital; positive to the anti-melanoma mab 9.2.27 [28].

HT-29: Colorectal cancer cell line, positive to anti-CRC mab c30.6 supplied through Dr R Ward, Garvin Institute.

SV174T: Colorectal cancer cell line, positive to anti-CRC mab 35A7, supplied by University Hospital Geneva.

MDA-MB-231: Breast cancer cell line, positive to uPA [29].

PC3, LN3: Prostate cancer cell lines supplied by the Prof P Russell, Prince of Wales Hospital, NSW Australia. PC3 is positive to uPA, LN3 positive to the mab J591 against the PSMA.

Flow cytometry was used to determine specificity of radio-conjugates for various cell lines.

2.4 Cell survival assay

With the exception of melanoma, for which the T-Thy method for DNA uptake was used [28], the MTS assay [30] was used to determine cell survival versus activity of the AIC or API.

2.5 In Vivo Model

Nude mice were used in all experiments. Mice received sc inoculations of 1-1.5 million cancer cells in the flanks (melanoma) or mammary pads (breast cancer). All experiments are approved by the ACEC on the UNSW.

2.5.1 Immunohistochemistry

Paraffin sections of tumour and lymph node tissues were stained with a human uPA mab (American Diagnostic Inc, # 394) as described [31].

3 Results

3.1 Radioisotope production and labeling

Ac:Bi generator technology has been developed to prepare and test several TAT approaches for melanoma [28,32]; leukaemia (Rizvi et al, preprint); colorectal cancer (Rizvi et al, preprint); breast cancer (Tian et al, preprint) and prostate cancer (Li et al, preprint). In addition, we have produced ^{149}Tb with a heavy ion accelerator and spallation source [22].

Labeling efficiencies of 95% were obtained for both cDTPAa and CHX-A" chelators, produced stable AIC and API with ~20% leaching after 2 half lives in serum; achieved 98% binding of cells with unlabelled antibody compared with 93-95% for the Bi-AIC [28].

The Ac:Bi generator allows us to produce Bi-213 at relatively low cost and in quantities sufficient to carry out *in vivo* experiments in mice for melanoma and breast cancer.

3.2 In Vitro

3.2.1 Melanoma

Using the T-Thy method for DNA uptake, the 37% uptake level occurs at 2 μCi for the ^{213}Bi -9.2.27 against the MM138 melanoma cells. This results compares with 286 μCi for the positron emitting ^{152}Tb -9.2.27 [28].

3.2.2 CRC

The Do (37% cell survival) value was calculated to be 2.0 μCi for the ^{213}Bi -35A7 [Rizvi et al preprint]. Free isotope was also studied for its cell killing abilities and the Do value was found to be 95 μCi . The Do values for ^{149}Tb labeled antibodies are 0.3 μCi and 1.2 μCi for c30.6 and 35A7 respectively, as compared to 31 μCi for the free ^{149}Tb (Fig 1). For ^{152}Tb , a positron emitter, the Do values were 230 μCi and 760 μCi for c30.6 antibody and the free ^{152}Tb , respectively.

3.2.3 Prostate Cancer

Studies have identified cell lines that express uPA (PC3) and PMSA (LN3). Cell survival experiments with the PC3 cell line (uPA+) show very high cytotoxicity with API as shown in (Fig 2), but negligible effect for controls.

3.3 In Vivo

3.3.1 Melanoma

In vivo results have shown that local TAT can completely inhibit the growth of sc melanoma (Fig 3) in nude mice with only 25 μ Ci injection at 2 days post-inoculation [34].

Further, large melanomas can be completely regressed by intra-lesional TAT of ~ 100 μ Ci. Results for several mice with sc melanoma are shown in Fig 4 at different stages of tumour regression after 200 μ Ci intra-lesional injection of ^{213}Bi -(9.2.27) AIC. There is no evidence of recurrence at 4 months.

3.3.2 Breast Cancer

Human breast cancer tumours xenografts were successfully induced in nude mice by inoculation 2×10^6 MDA-MB-231 cells into mammary fat pad of nude mice. TAT can completely inhibit the growth of sc breast cancer (Fig 5) in nude mice with only 25 μ Ci injection at 2 days post-inoculation.

Sections of breast cancer xenografts are stained with mab against uPA and shown in Fig 6. Invasion of the regional lymph nodes by the breast cancer cells is clearly seen (arrow), thus establishing a genuine metastatic cancer model.

Visible xenografts appear around 2-3 weeks. After 4-5 weeks, cancer cells were identified histologically in sections taken through primary tumour tissue and in the axillary and cervical lymph nodes (Fig 6a,b). The positive uPA antigen expression on primary and metastatic cancer cells were identified by immunohistochemistry, as shown

in Fig 6c,d. Paraffin sections were stained with uPA monoclonal antibody #394 (brown colour) to identify the uPA antigen positive cancer cells.

However, unlike melanoma, intralesional TAT was not able to regress established tumours, but in all cases a partial response was observed.

3.3.3 Toxicology

Our toxicology studies show that nude mice can tolerate up to 3 mCi/kg of alpha chelate (AIC, API) by systemic administration, causing a 5-10% weight loss. Up to 10 mCi/kg of AIC via intra-lesional injection causes <20% weight loss.

4 Conclusions

In vitro results in this study show highly selective cytotoxicity to targeted cancer cells. The in vivo data show that complete inhibition of tumourogenesis is possible for both melanoma and breast cancer. Intralesional injection of larger melanomas can achieve complete regression without recurrence, but this was not the case for breast cancer tumours.

These successful results for melanoma point to the potential application of local TAT in the management of melanoma (re-excision of positive margin melanoma, Hutchinson's Melanotic Freckle where surgery is undesirable, and recurrent melanoma). Anti-tumourogenesis has implications for the control of subclinical micrometastatic cancer after excision of a high risk primary tumours or isolated limb infusion, and ultimate control of early stage micrometastases.

Breast cancer results show that the in vivo sc mammary pad model is a realistic metastatic model for lymphatic invasion, with the malignant cells expressing uPA. However, results for intra-lesional injection are less encouraging, possibly because of poor diffusion of conjugate through the breast cancer tumour.

This study shows that TAT may well be an efficacious and safe therapeutic modality and that AICs and API may be able to address the problems of isolated and small cell cluster toxicity, preangiogenic lesions and variable antigenic expression.

Problems relating to uptake time in tumours are not applicable in this application as blood flow quickly carries the alpha chelate to microscopic lesions. TAT is not indicated for solid tumours, except by direct, intra-lesional injection as in the case for melanoma.

5 Acknowledgements

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6 Figure Captions

Fig 1 Cell survival after incubation with Tb-149 AICs in CRC cells, compared with free Tb-149.

Fig 2 Cell survival after incubation with ^{213}Bi -API in PC3 prostate cancer cells, compared with non-specific AIC.

Fig3 Inhibition of melanoma by local TAT. Tumour area vs post-inoculation time shown for control, 25 μ Ci non-specific AIC, 12.5 μ Ci specific AIC and complete inhibition with 25 μ Ci specific AIC at 2 days post-inoculation.

Fig 4 Stages of melanoma regression in different mice after intra-lesional injection of 200 μ Ci of ^{213}Bi -AIC.

Fig 5 Inhibition of breast cancer by alpha-PAI2, showing tumour area vs post-inoculation time for control (PBS), 12.5 μ Ci and complete inhibition for 25 and 50 μ Ci alpha-PAI2 for sc injection at 2 days post inoculation. 2×10^6 MDA-MB-231 cells were Inoculated sc into the mammary fat pad on both sides of 4-6 week nude mice.

Fig 6 Histopathological sections showing cancer cells in xenografted primary breast tumours and metastatic lymph nodes of nude mice. The brown colour identifies uPA antigen positive cancer cells. (A) primary tumour (HE stain, x 100). (B) primary tumour (+uPA, x 400). (C) metastatic lymph node (+uPA, x 100). (D) metastatic lymph node (+uPA, x 200). Black arrows indicated cancer cells.

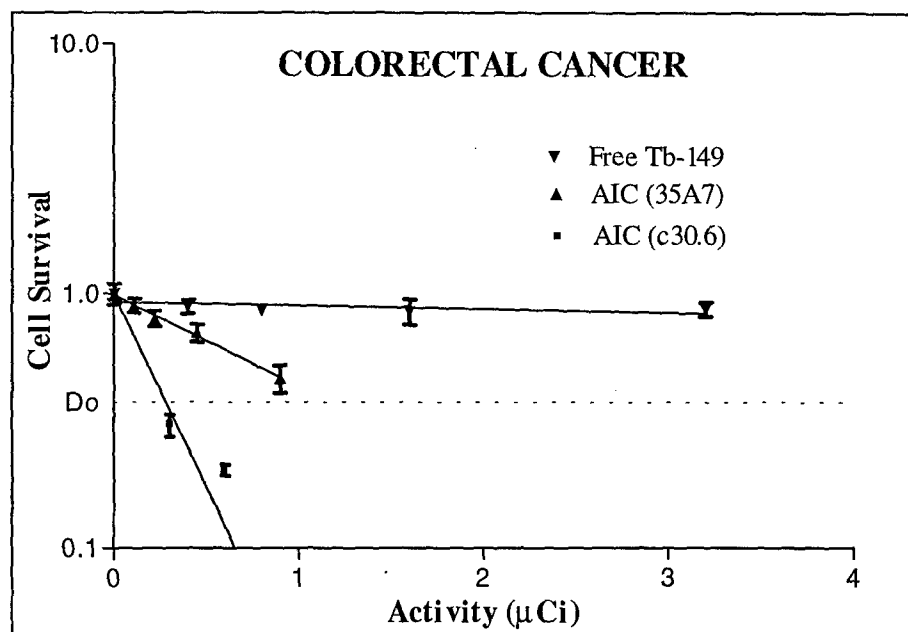


Fig 1 Cell survival after incubation with Tb-149 AICs in CRC cells, compared with free Tb-149.

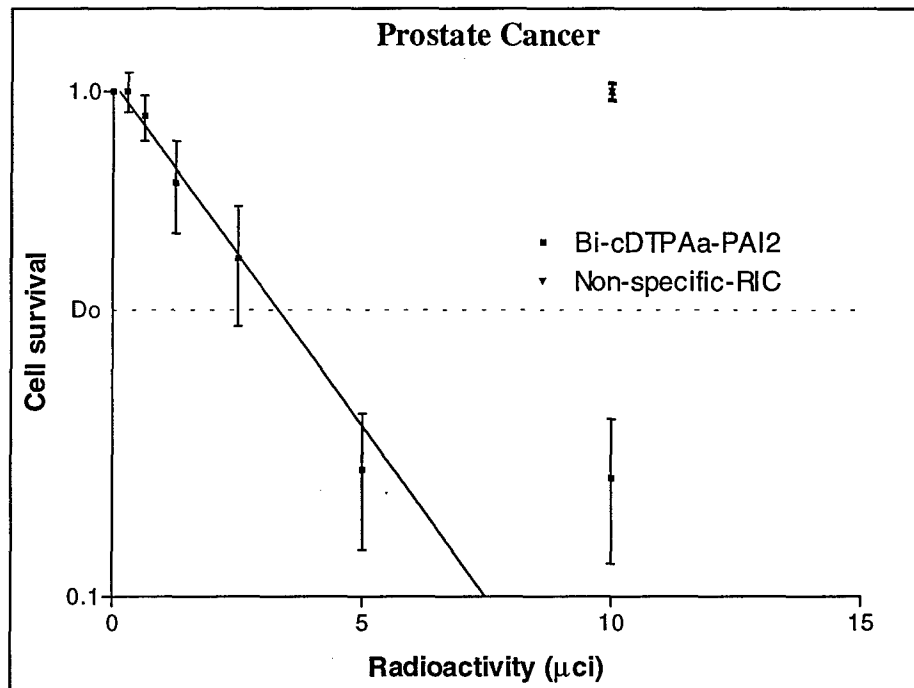
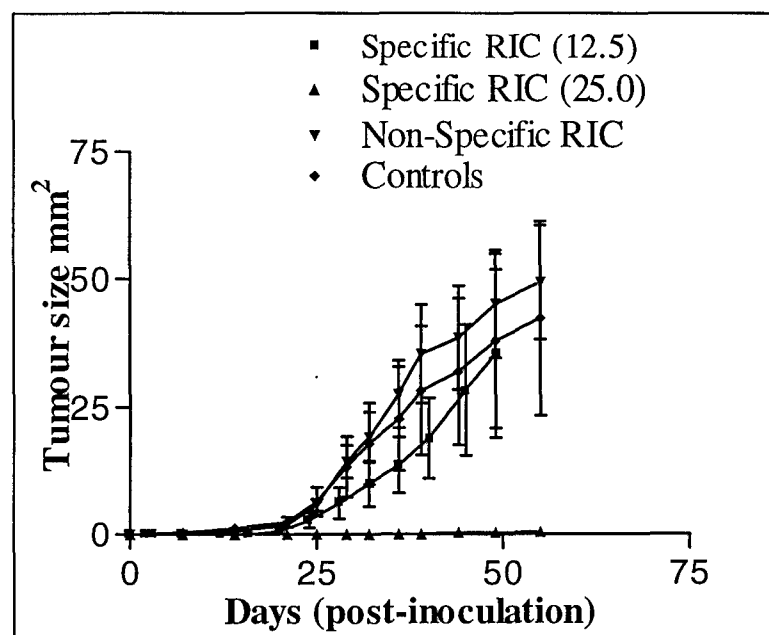


Fig 2 Cell survival after incubation with ^{213}Bi -API in PC3 prostate cancer cells, compared with non-specific AIC.

Fig3 Inhibition of melanoma by local TAT. Tumour area vs post-inoculation time shown for control, 25 μ Ci non-specific AIC, 12.5 μ Ci specific AIC and complete inhibition with 25 μ Ci specific AIC at 2 days post-inoculation.



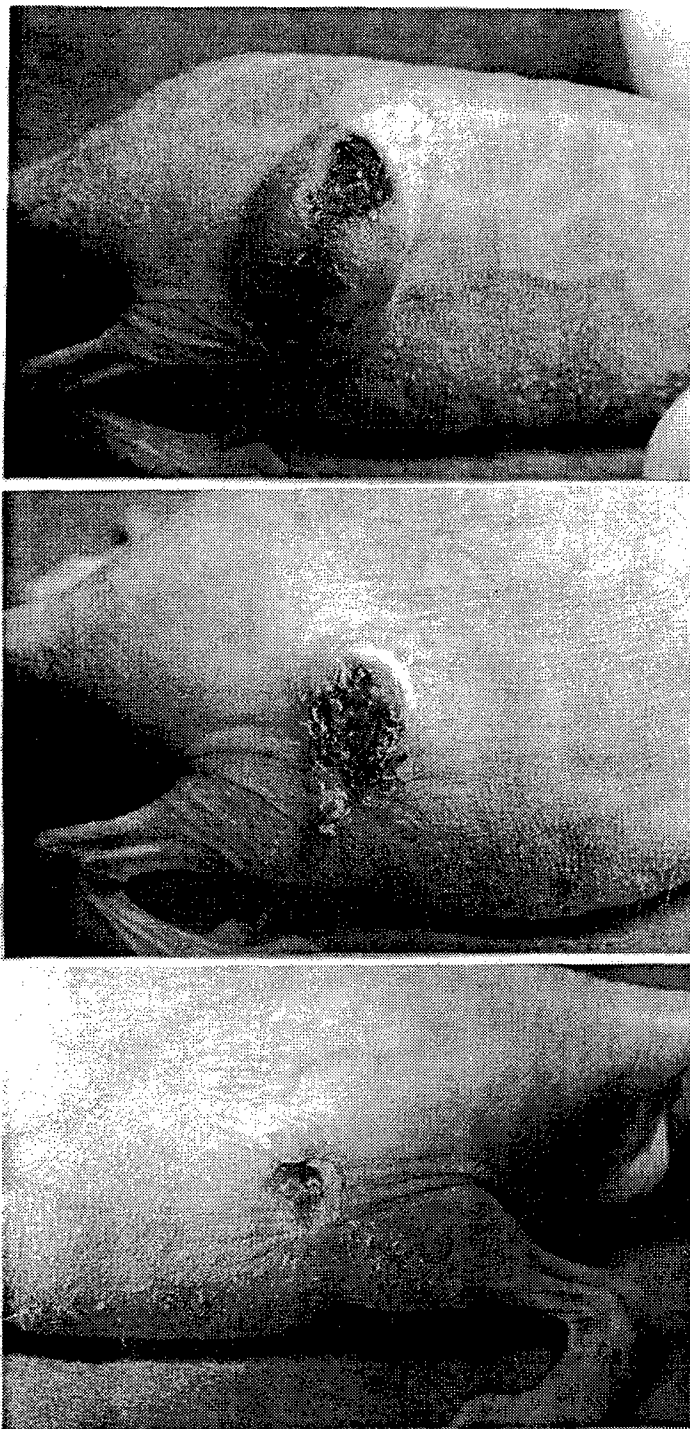
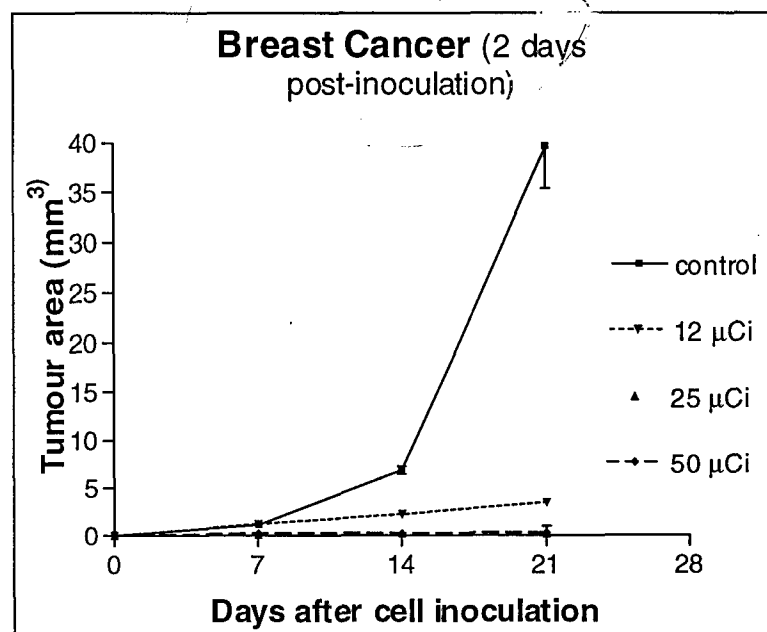


Fig 4 Stages of melanoma regression in different mice after intra-lesional injection of 200 μCi of ^{213}Bi -AIC.

Fig 5 Inhibition of breast cancer by alpha-PAI2, showing tumour area vs post-inoculation time for control (PBS), 12.5 μCi and complete inhibition for 25 and 50 μCi alpha-PAI2 for sc injection at 2 days post inoculation. 2×10^6 MDA-MB-231 cells were inoculated sc into the mammary fat pad on both sides of 4-6 week nude mice.



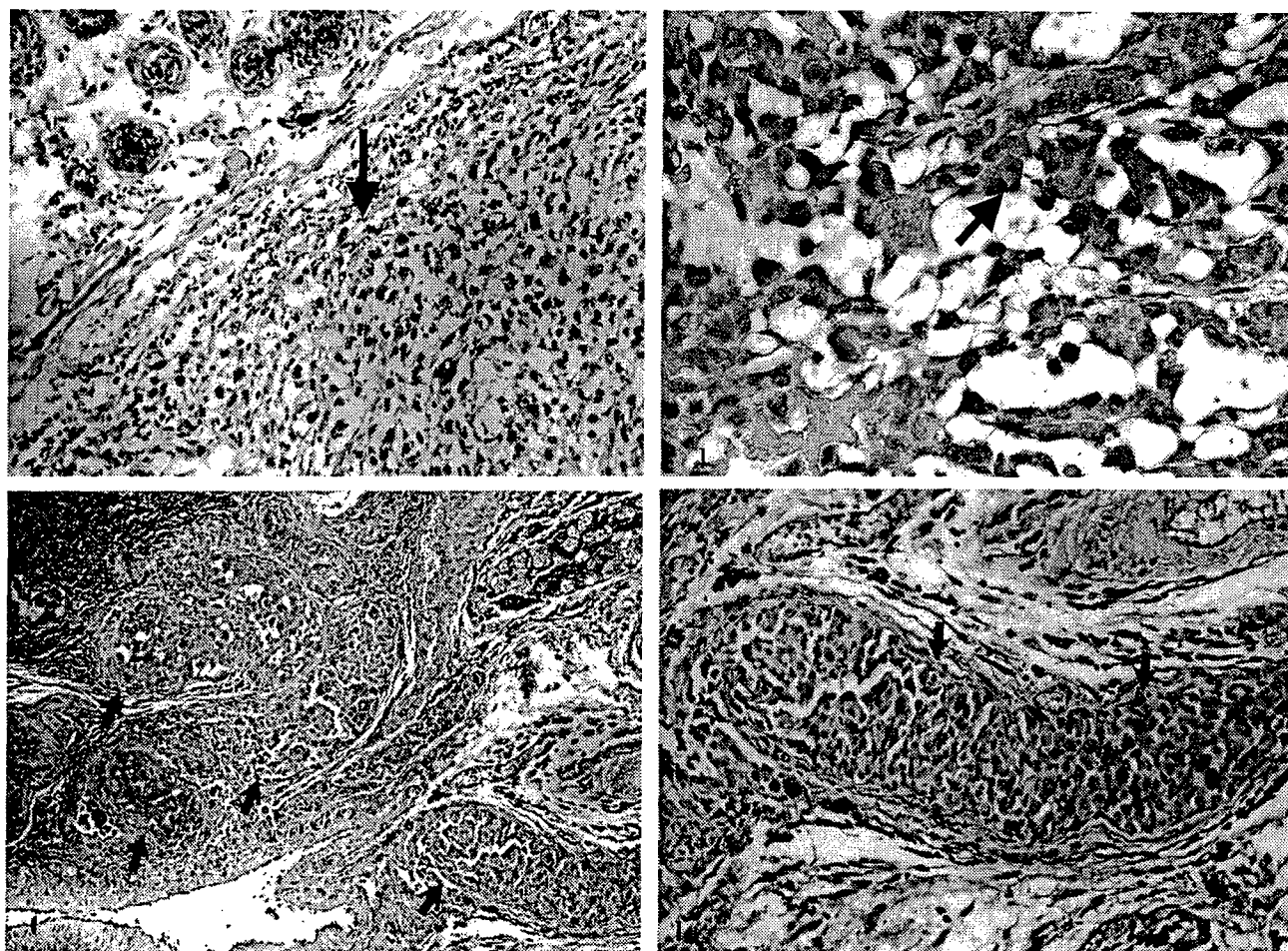


Fig. 6 Histopathological sections showing cancer cells in xenografted primary breast tumours and metastatic lymph nodes of nude mice. The brown colour identifies uPA antigen positive cancer cells. (A) primary tumour (HE stain, x 100). (B) primary tumour (+uPA, x 400). (C) metastatic lymph node (+uPA, x 100). (D) metastatic lymph node (+uPA, x 200). Black arrows indicated cancer cells.

A METHOD OF TREATMENT AND AGENTS FOR USE THEREIN

The present invention relates to a method of treating a condition in a mammal, which condition is characterised by the undesirable, detrimental or otherwise unwanted growth of uPAR expressing cells and agents useful for same. More particularly, the present invention contemplates a method of treating said condition by specifically targeting the subject cells utilising plasminogen activator inhibitor or functional derivative, equivalent, homologue, analogue or mimetic thereof coupled to a toxin such as an alpha particle emitting radioisotope. The method of the present invention is useful, *inter alia*, in the targeted treatment of conditions such as neoplasms and, in particular, metastatic cancers.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The acquisition of the malignant phenotype involves a number of initiation and progression factors linked together in a multistep process (Meyer and Hart 1998; Haber and Fearon, 1998). Malignant tumours are potentially lethal because of the ability of cells within the tumour to invade and spread (metastasise) throughout the body (Meyer and Hart 1998). The process of metastasis can be summarised in the following steps; escape of tumour cells from a primary tumour mass and invasion into surrounding histologically normal tissue, intravasation and extravasation (entry into and exit from the vasculature or lymphatic system), and growth and survival (via angiogenesis) of tumour cells at a secondary site. One of the major failures in the treatment of cancer is poor detection and eradication of metastases before vital organ functions are compromised, resulting in minimal long-term survival benefit (Allen, 1999). Thus, effective cancer treatment entails

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primary tumour resection followed by removal of all metastases arising from the primary.

The latter would require (1) identification of a marker specific to metastatic cells, and (2) development of a ligand that can be used to specifically target and kill the cells expressing the marker.

Proteolytic enzymes such as urokinase plasminogen activator (herein referred to as "uPA") play a role in tumour angiogenesis and metastatic cell migration; both of which are processes that require tissue barriers to be breached (reviewed in Andreasen *et al.*, 1997).

Under normal physiological conditions, most cells express very little or no uPA (Pollanen *et al.*, 1991). Urokinase plasminogen activator converts zymogen plasminogen into the highly active protease plasmin, which has broad specificity towards integral extracellular matrix (ECM) molecules (eg: type IV collagen, vitronectin, proteoglycan, fibronectin and laminin) (Pollanen *et al.*, 1991). Plasmin also contributes to ECM remodelling by activating zymogen metalloproteases (MMPs) which more thoroughly degrade the collagen structural components (Pollanen *et al.*, 1991). While plasminogen can also be converted to plasmin by tissue plasminogen activator (tPA), tPA is primarily responsible for fibronolysis (Lijnen and Collen, 1982; Pollanen *et al.*, 1991). In contrast uPA is primarily involved in pericellular proteolysis as it binds to its specific cell-surface receptor uPAR (Pollanen *et al.*, 1991). The activities of uPA and plasmin are physiologically inhibited by the serpins plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2) and alpha 2-antiplasmin, respectively (Pollanen *et al.*, 1991). The uPA system and MMPs, such as MMP-9, have been shown to act cooperatively in allowing tumour cells to breach the vascular wall (Kim *et al.*, 1999).

Prior art "magic bullet" style treatments for neoplastic conditions have been extensively investigated but, to date, have met with little or no success.

Specifically, most previous attempts at such targeted treatment have relied on the use of antibodies directed to various cell surface molecules expressed by cancer cells. However, such approaches have suffered from many drawbacks including:

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- (i) the surface molecules to which the antibodies are directed have not been uniquely expressed by the cancer cells. Accordingly, such treatments have also been toxic to a significant number of normal cells.
- (ii) the antibodies which have been utilised in such treatments have been of mouse origin or have been "humanised" mouse antibodies. The use of such antibodies has led to immunological complications associated with the HAMA response. That is, repeated dosing of humans with murine antibodies, such as humanised antibodies, has led to the development of anti-murine antibodies thereby causing both rapid clearance of the murine antibodies and the production of immune complexes which cause the HAMA response.
- (iii) the antibody-cell surface molecule complexes are often internalised. Accordingly, the toxin which is coupled to the subject antibody is less effective.
- (iv) the strength of binding of most antibodies to a target cell surface molecule is low with a dissociation constant of approximately 10^{-6} M being common.
- (v) in coupling a toxin to the antibody the point of linkage cannot usually be predicted. Depending on the structure of a given antibody, coupling may occur at the antigen binding site region of the antibody thereby rendering the antibody useless.

Further, prior art radiocolloid therapy is not suitable for adjunctive therapy as it is not selective of cancer cells. To the extent that beta and gamma emitting radionuclides have been coupled to specific monoclonal antibodies, problems have been experienced with most of the dose leaving the cancer cell. Therefore, therapeutic doses cannot be achieved without inducing severe complications.

Accordingly, there is a need to develop a more effective method of targeting neoplastic cells for treatment, which method provides both improved selectivity in terms of its targeting function and improved delivery of a toxic signal. In terms of the delivery of a

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toxic signal, there is a need to develop a method which provides both a maximal dose of the subject toxin to the target cell but with minimal impact upon proximally located non-target cells.

In work leading up to the present invention the inventors have determined that plasminogen activator inhibitors (herein referred to as "PAI") and in particular PAI-2, can be used as a targeting molecule for specific delivery of a toxin since cancer cells express the uPA/uPAR complex while non-diseased cells express little or no uPA/uPAR complex. Further, the inventors have determined that the coupling of an alpha particle emitting radioisotope to PAI-2 does not inhibit binding of PAI-2 to uPA and still further, that the labelled PAI-2-uPA/uPAR complex is internalised by the targeted cell thereby providing maximal impact of the high energy radioactive emission on the target cell and minimal impact on proximal cells. This is due to the alpha-emitter being highly toxic over a short range only. Finally, unlike the observed dissociation constant of 10^{-6} M with respect to antibody/antigen interactions, coupling of PAI-2 to uPA is extremely strong, exhibiting a dissociation constant in the order of 10^{-11} M, thereby minimising dissociation of the radiolabelled PAI-2 and consequently decreasing the risk of a toxic impact on localised non-target cells.

Accordingly, one aspect of the present invention is directed to a method of treating a condition in a mammal, which condition is characterised by the undesirable, detrimental or otherwise unwanted growth of cells expressing a uPA/uPAR complex, said method comprising administering to said mammal an effective amount of PAI or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI is bound, linked or otherwise associated with a toxin, for a time and under conditions sufficient to down-regulate the growth of said cells.

Reference to "PAI" should be understood as a reference to any PAI or functional derivative, equivalent, homologue, analogue or mimetic thereof. In this regard, the PAI may be of any suitable form, such as a mature molecule, a precursor form of said mature molecule, mutant, polymorphic variant or a derivative, homologue, equivalent, analogue

or mimetic thereof which exhibits at least one of the functional activities of said PAI. In a preferred embodiment, said PAI is PAI-2, a glycoprotein of the serine protease inhibitor type and which exists in both glycosylated and unglycosylated forms. (Andreasen, 1990). Without limiting the present invention in any way, PAI-2 exhibits several advantages over PAI-1. First, PAI-2 is very stable in vitro compared to PAI-1 which is oxidation sensitive and easily inactivated (Kruithof et al., 1995). Secondly, PAI-2 is approximately 10,000 fold less active towards t-PA than PAI-1 and would not lead to the side effect that fibrinolysis is inhibited. Thirdly, high blood levels of PAI-2 are thought less likely to cause any other adverse side effects since high levels of PAI-2 are found during late pregnancy (in the non-pregnant state blood levels of PAI-2 are not detectable) and are not associated with toxicity (Kruithof et al., 1996).

The present invention therefore more particularly provides a method for treating a condition in a mammal, which condition is characterised by the undesirable detrimental or otherwise unwanted growth of cells expressing a uPA/uPAR complex, said method comprising administering to said mammal an effective amount of PAI-2 or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI-2 is bound, linked or otherwise associated with a toxin, for a time and under conditions sufficient to down-regulate the growth of said cells.

Reference to "toxin" should be understood as a reference to any suitable toxin which achieves the object of providing a signal which reduces, prevents or otherwise inhibits the proliferation, differentiation or maintenance of subject cell (herein referred to as "down-regulating the growth" of said cell). The subject toxin may act by a variety of means including providing its signal via direct contact with a subject cell or emitting a molecule or particle, such as radiation in the case of a radioactive isotope toxin, which provides the signal to the subject cell. Preferably the toxin is a radioisotope and even more preferably a radioisotope which is highly toxic over a short range and exhibits a short half life thereby minimizing the occurrence of inadvertent toxicity on proximally located non-target cells. Most particularly, said radioisotope is an alpha particle emitting radioisotope. Examples of alpha-emitting radioisotopes suitable for use in the method of the present

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invention include, but are not limited to, Tb-149 or Bi-213. It should be understood that the toxin which is utilised in the method of the present invention may be in a purified, partially purified or unpurified form. It may also form a component of a larger molecule. The toxin may be naturally occurring or it may be synthetically or recombinantly produced.

According to this preferred embodiment, the present invention provides a method of treating a condition in a mammal, which condition is characterised by the undesirable, detrimental or otherwise unwanted growth of cells expressing a uPA/uPAR complex, said method comprising administering to said mammal an effective amount of PAI-2 or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI-2 is bound, linked or otherwise associated with an alpha particle emitting radioisotope or functional derivative, equivalent, homologue, analogue or mimetic thereof, for a time and under conditions sufficient to down-regulate the growth of said cells.

Still more preferably said alpha-emitting particle emitting radioisotope is Tb-149 or Bi-213.

As detailed above, reference to "growth" of a cell should be understood as a reference to the proliferation, differentiation and/or maintenance of viability of the subject cell, while "down-regulating the growth" of a cell is a reference to reducing, preventing or inhibiting the proliferation, differentiation and/or maintenance of viability of the subject cell. In a preferred embodiment the subject growth is proliferation and the subject down-regulation is killing. In this regard, killing may be achieved either by delivering a fatal hit to the cell or by delivering to the cell a signal which induces the cell to apoptose.

The present invention thereby preferably provides a method for treating a condition in a mammal, which condition is characterised by the undesirable, detrimental or otherwise unwanted proliferation of cells expressing a uPA/uPAR complex, said method comprising administering to said mammal an effective amount of PAI-2 or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI-2 is bound, linked or otherwise associated with an alpha particle emitting radioisotope or functional derivative,

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equivalent, homologue, analogue or mimetic thereof, for a time and under conditions sufficient to kill said cells.

In this regard, "treatment" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a mammal is treated until total recovery. Accordingly, "treatment" includes amelioration of the symptoms or severity of a particular condition or preventing or otherwise reducing the risk of developing a particular condition.

The present invention is directed to inhibiting the unwanted growth of cells expressing a uPA/uPAR complex. This should be understood as a reference to cells expressing uPAR (the uPA receptor) to which is coupled a uPA molecule. The uPA molecule which has bound to the uPAR may be derived from any source and has not necessarily been produced from the subject cell in an autocrine fashion - although it should be understood that this possibility is not excluded. Without limiting the present invention to any one theory or mode of action, PAI-2 (to which has been coupled a toxin) will interact with the uPA molecule which, in turn, has interacted with the uPAR expressed by the subject cell.

The PAI-2 is thereby specifically targeted to cells which express a uPAR to which is bound uPA. Still without limiting the present invention in any way, uPAR is expressed by neoplastic cells and is not expressed at significant levels by non-neoplastic cells.

Reference to "interact" should be understood as a reference to any form of interaction. Said interaction may occur via the formation of bonds such as covalent bonds, hydrogen bonds, van Der Waals forces or via any other mechanism of interaction.

The present invention is therefore preferably directed to a method of treating a neoplastic condition in a mammal, said method comprising administering to said mammal an effective amount of PAI-2 or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI-2 is bound, linked or otherwise associated with a toxin for a time and under conditions sufficient to down-regulate the proliferation of the subject neoplastic cells.

Preferably, said toxin is an alpha particle emitting radioisotope and even more preferably Tb-149 or Bi-213.

Reference to "neoplastic cell" should be understood, in the context of the present invention, as a reference to a cell exhibiting abnormal growth (as hereinbefore defined) and which cell expresses uPAR. The neoplastic cell may be a benign cell or a malignant cell. Preferably, the cell is malignant. Without limiting the present invention in any way, uPAR is overexpressed in metastatic cancers including, but not limited to, breast cancer, prostate cancer and colorectal cancer.

The present invention therefore preferably provides a method of treating a metastatic cancer in a mammal, said method comprising administering to said mammal an effective amount of PAI-2 or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI-2 is bound, linked or otherwise associated with a toxin, for a time and under conditions sufficient to down-regulate the proliferation of the subject cancer cells.

"Functional derivatives and mimetics" include fragments, parts, portions, mutants, and mimetics from natural, synthetic or recombinant sources including fusion proteins exhibiting any one or more of the functional activities of the subject PAI or toxin. To the extent that the subject PAI or toxin is a protein, derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. An example of substitutional amino acid variants are conservative amino acid substitutions. Conservative amino acid substitutions typically include substitutions within the following

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groups: glycine and alanine; valine, isoleucine and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

Homologues of a PAI or toxin contemplated herein include, but are not limited to, molecules derived from different species.

Chemical and functional equivalents of PAI or toxin should be understood as molecules exhibiting any one or more of the functional activities of PAI or toxin, respectively, and may be derived from any source such as being chemically synthesized or identified via screening processes such as natural product screening.

The derivatives of PAI or toxin include fragments having particular epitopes of parts of the entire PAI protein or toxin fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, PAI or derivative thereof may be fused to a molecule to facilitate its delivery to a cell.

"Analogues" of PAI or toxin contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH_4 .

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The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 1.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornyl- carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine		L-N-methylglutamine	Nmgln
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
D-alanine	Dal	Chexa L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Das	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
D-threonine	Dthr	L-N-methylethylglycine	Nmetg
D-tryptophan	Dtrp	L-N-methyl-t-butylglycine	Nmtbug
		L-norleucine	Nle
		L-norvaline	Nva

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D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
D-valine	Dval	α -methyl- γ -aminobutyrate	Mgabv
D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- α -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
D- α -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis

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D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- α -methylalanine	Mala
L- α -methylarginine	Marg	L- α -methylasparagine	Masn
L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
L- α -methylserine	Mser	L- α -methylthreonine	Mthr
L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe

1-carboxy-1-(2,2-diphenyl-Nmbc
ethylamino)cyclopropane

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with $n=1$ to $n=6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

Without limiting the present invention to any one theory or mode of action, it is thought that PAI-2 exhibits high selectivity to neoplastic cells at their most malignant phase. In this regard, PAI-2 functions as a "homing molecule" for cancer cells which express uPA, bound to uPA receptors, on their surface. Upon binding of radiolabelled PAI-2 to the cell surface uPA/uPAR complex, the entire molecular complex is internalised. Once internalised, the high energy radioactive emission kills the subject cell with little or no effect on proximally located non-target cells. Due to the absence of such side effects, together with the short half-life and rapid decay of the selected alpha particle emitting radioisotope, there is little or no detrimental effect to the subject as a whole. The high turnover cell toxicity of radiolabelled PAI-2 is thought to derive from the tight, essentially irreversible binding of PA-2 to uPA.

Still without limiting the present invention in any way, Tb-149 and Bi-213, in particular, chelate to PAI-2 to form very stable bonding. In this regard, any suitable method may be utilised to achieve chelation. Preferably, the chelators cDTPAa and CHX-A are utilised. Alpha radiation can then kill the subject cancer cells in 1-5 nuclear hits. The linear energy transfer (LET) for the α particle, being very much greater than that for the beta rays, causes a high relative biological effectiveness over a much shorter range. As a result, a much greater fraction of the total energy is deposited in cells with alphas and very few nuclear hits are required to kill a cell (Lloyd *et al.*, 1979, Kassis *et al.*, 1986). Further, the short half life of alpha-emitters is particularly suitable for the killing of cancer cells and pre-angiogenic lesions while simultaneously releasing only a low

radiation dose to normal tissue. The alpha therapeutic ratio is thought to be two orders of magnitude greater than that for a high energy beta emitter and is therefore the preferred form of toxin for use in the method of the present invention.

Administration of the toxin labelled PAI, in the form of a pharmaceutical composition, may be performed by any convenient means. The toxin labelled PAI of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the toxin chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 μg to about 10 mg of toxin labelled PAI may be administered per kilogram of body weight per day. For example from about 0.1 μg -5 mg, 10 μg -5 mg or 100 μg -1 mg. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The toxin labelled PAI may be administered in any convenient manner such as by the intravenous, intraperitoneal, intramuscular, subcutaneous or intradermal. Preferably, the toxin-labelled PAI is administered intravenously.

In accordance with these methods, the toxin labelled PAI defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

The term "mammal" should be understood as a reference to a human, primate, livestock animal (eg. sheep, pig, cow, horse, donkey) laboratory test animal (eg. mouse, rat, rabbit, guinea pig) companion animal (eg. dog, cat) or captive wild animal (eg. fox, kangaroo, deer). Preferably, the mammal is a human.

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An "effective amount" means an amount necessary to at least partly attain the desired response.

Another aspect of the present invention contemplates a method of down-regulating the growth of cells expressing a uPA/uPAR complex, said method comprising administering to said mammal an effective amount of PAI or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI is bound, linked or otherwise associated with a toxin.

Preferably said PAI is PAI-2 and said toxin is an alpha particle emitting radioisotope.

Still another aspect of the present invention relates to the use of PAI or functional derivative, equivalent, homologue, analogue or mimetic thereof, which PAI is bound, linked or otherwise associated with a toxin, in the manufacture of a medicament for the treatment of a condition in a mammal, which condition is characterised by the undesirable, detrimental or otherwise unwanted growth of cells expressing a uPA/uPAR complex.

Preferably said PAI is PAI-2 and said toxin is an alpha-particle emitting radioisotope.

Even more preferably said condition is a neoplasm and still more preferably a metastatic malignancy.

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising PAI or functional derivative, homologue, analogue, chemical equivalent or mimetic thereof, which PAI is bound, linked or otherwise associated with a toxin, together with one or more pharmaceutically acceptable carriers and/or diluents. Preferably, said PAI is PAI-2 and said toxin is an alpha particle emitting radioisotope. The toxin labelled PAI molecules are referred to as the active ingredients. The pharmaceutical composition is preferably designed for intravenous application.

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The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimersal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active

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substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.1 μg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.1 μg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The present invention is further described by the following non-limiting Examples.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1** Formation of SDS-stable complex of radio-conjugated PAI-2 with UPA, indicating activity of PAI-2;
- Figure 2** Purity of radio-conjugated PAI-2 in ITLC;
- Figure 3** MDA-MB-231 cell survival with radio-conjugate PAI-2;
- Figure 4** MCF-7 cell survival with radio-conjugate PAI-2;
- Figure 5** Confocal microscopy of endocytosis of radio-conjugate PAI-2 by MDA-MB231 cells;
- Figure 6** Tolerance of radio-conjugate PAI-2 by nude mice;
- Figure 7** Breast Cancer in vivo model. 25 Ci radio-conjugate PAI-2 injected 2 days after tumor cells;
- Figure 8** Breast Cancer in vivo model. 50 Ci radio-conjugate PAI-2 injected 2 days after tumor cells;
- Figure 9** Breast Cancer in vivo model. 25 Ci radio-conjugate PAI-2 injected 7 days after tumor cells;
- Figure 10** Breast cancer in vivo model. 25 Ci radio-conjugate PAI-2 injected 14 days after tumor cells.

EXAMPLE 1

PREPARATION OF PAI-2 ALPHA-EMITTER CONJUGATE

Production

The rare earth nuclide ^{149}Tb is produced on a tandem, cyclotron or linear accelerator using high energy heavy ions such as boron or carbon or nitrogen ions to bombard targets of Praseodymium, eg. $\text{Pr}(^{12}\text{C}, 4\text{n})$ or Neodymium $\text{Nd}(^{12}\text{C}, 5\text{n})$ at higher energies. Metal targets are rolled and mounted on a frame. A thin target has 1mg cm^{-2} , a thick target has 30 mg cm^{-2} . A catcher foil is used to collect Tb ions in the thin foil geometry.

Tb-149 is also produced via the spallation reaction of high energy charged particles, eg. protons, on a high atomic number target such as tantalum. The ions are passed through a magnetic mass analyser to select the $A=149$ component of the yield. The Tb-immunoconjugates are made using the longer lived isotope Tb-152 as an exact analogue.

An alternative α radiolabel is Bi-213, which is produced by α decay from Ac-225. the Bi-213 is eluted from the Ac-225 with $250\mu\text{L}$ of fresh 0.15M HI followed by $250\mu\text{L}$ water. The activity of the Bi-213 is assayed against the Au-198 setting in the dose calibrator. The first elution is not used as it contains cold Bi.

Purification

In the production of Tb-149, the product nuclides are separated from the thick target by dissolution in 6M nitric acid, the sample is irradiated to dryness and yield determined by gamma ray spectroscopy. The residue is dissolved in 0.16M γ -hydroxyisobutyric acid and passed through a cation exchange column (particle size $13\mu\text{m}$). The pH of the eluant is adjusted to 5 by aqueous ammonia. Elution was under a pressure of 7kg cm^{-2} at a flow rate of 0.5mL min^{-1} . Terbium fractions are dried gently and heated to 450 degrees to destroy the Tb-isobutyrate complex. The residue is dissolved in dilute nitric or hydrochloric acid for the radiolabeling procedure.

Bi-213 is eluted with 0.15M hydriodic acid and the pH of the eluant adjusted as above.

Labelling

The purified product is chelated to molecules which target specific cancer cells. A number of different chelation procedures are available in the literature which use cDTPAa, DTPA-CHX", DOTA (1,4,7,10-tetraazacyclododecane-N,N,N,N,tetraacetic acid), and TETA.

The labelling procedure IS a modification of the method used by Izard *et al* (1992). Briefly, both chelators ie. cDTPAa and CHX-A" were prepared in chloroform and were purified under a stream of Nitrogen. A chelator:protein molar ratio of 20:1 and 4:1 was maintained for cDTPAa and CHX-A" respectively. After a 45-minute on-ice incubation of the chelator and PAI2 (Biotech Australia), the conjugate was purified on a PD-10 column (Pharmacia Biotech) using 0.5 M sodium acetate at pH 5.5 as the eluting buffer. This was followed by the addition of Bi-213 and TB-149 or TB-152). After 20-minute incubation, the ratio-conjugate (RI) was again purified on another PD-10 column using PBS at pH 7.0 as the eluting buffer. The protein recovery is >97% as determined by instant thin layer chromatography (ITLC) of the fractions obtained. A similar procedure was followed for labelling the non-specific protein, bovine serum albumin (BSA).

The inhibitory activity of cDPTAa-conjugated PAI2 was confirmed by its ability to form complexes after 40 min incubation at 20°C with active uPA. Complex formation was detected by a molecular mass shift by SDS-PAGE (12% non-reducing gel)(Figure 1).

Stability of radioconjugated protein

The radioconjugates were incubated with fresh human serum at 37°C for 45 min (equivalent to one half-life for bi-213). Samples were then analysed by ITLC (Figure 2). Radioconjugates remain at the origin whereas any free label runs at the solvent front.

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Similar experiments have been performed with Tb radioconjugates over long time periods.

EXAMPLE 2

IN VITRO ANALYSIS OF PAI-2 ALPHA EMITTER CONJUGATE

Tb-149 and Bi-213 ARCs have been produced and their stability, labelling efficiency, targeting and in vitro cell survival (37% survival for 2 μ Ci) with MDA-MB-231 and MCF-7 breast cancer cell lines have been tested. The following ARCs have been prepared, by the inventors, for the first time:

Tb-149.cDTPAa.PAI2

Tb-14.CHX.PAI2

Bi-213.cDTPAa.PAI2

Bi-213.CHX.PAI2

The method of the present invention is suitable for any early stage metastatic cancer which express high levels of uPA-uPAR.

Cell survival

Cell survival data have been obtained for two cell lines (MDA-MB-231 and MCF-7) in the presence of plasminogen with or without the specific uPA activity blocking agent {gly-gly-arg chloromethylketone (EGR-CMK), as this agent effectively inhibits PAI2 binding; Hang *et al.* 1998}. The 37% survival dose ($D_{0.37}$) values are shown in Table 1.

Conclusions:

a) EGR-CMK significantly improves survival (by a factor of 2.5, Table 1) as a result of inhibition of the PAI2 interaction with cellular uPA, proving the specific cytotoxicity of α -PAI2.

b) MDA-MB-231 and MCF-7 have similar survivals (ie. about $2\mu\text{Ci}$ for 37%), as shown in Figures 3 and 4. This does not imply similar cellular uPA levels but rather the high toxicity of the α -PAI2.

c) No cell killing was observed with the freshly isolated normal human leukocytes (Table 1) reflecting that non-targeted cells are immune from α -PAI2.

Endocytosis

The α -PAI2 complex is endocytosed in breast cancer cells, as shown by confocal microscopy in Figure 5. Some cells shown in Figure 5 have been stably transfected with the green fluorescent protein (GFP). This uncloned population was then incubated with TRITC- (red fluorescence) labeled PAI2 ($10\mu\text{g/ml}$) for 1 h at room temperature before being viewed under the confocal microscope. The red fluorescent spots are evidence of internalisation and accumulation into vesicles (probably endosomes and lysosomes). Internalisation improves the cytotoxicity for isolated cells as the probability for α tracks crossing the nucleus is increased.

EXAMPLE 3

IN VIVO ANALYSIS OF PAI-2 ALPHA EMITTER CONJUGATE

A-PAI2 has been administered by intra-peritoneal injection in adult (10-12 weeks), male BALB/c nude mice at a dose of 3mCi/kg (ie. 3 times the accepted tolerance dose in humans for an α -labeled antibody; D. Scheinber, Memorial Sloan Kettering Institute, personal communication to BA). A 10% short term weight loss was observed in 2 of 3 mice (α -PAI2-i and -iii), with recovery by day 11 (Figure 6). The third mouse (α -PAI2-ii) lost approximately 10% body weight at day 7 and is stable at this point of the study. Cold chelated-PAI2, cold PAI2 and the control (PBS) mouse all showed small variations in weight. Overall, the mice were clinically unaffected for the time period observed so far.

Clinical protocol

High risk patients are treated with the Tb-149 or Bi-213-ARCs immediately after detection and removal of the primary tumour or at the minimal residual disease stage to selectively kill isolated cancer cells or small nests of such cells as at the preangiogenic stage, where rapid uptake and incorporation can occur. Concomitant dose to normal tissue is much lower than for beta radionuclides, leading to improved prognosis.

Identification of a specific target of cancer (the targeting agent)

Targeting uPA-overexpressing cells using its natural inhibitor PAI2 linked to radioisotopes remains an unexploited mode of attack against cancer malignancy. The inventors have established the pharmacokinetics and biodistribution of exogenously administered human recombinant radioiodinated (^{125}I) PAI2 in both control and tumour-bearing animals (Hang *et al.*, 1998). Briefly, these data show that there is a difference in clearance and organ uptake between control animals and those bearing human colon cancer (HCT116 cell line) xenografts expressing moderate amounts of receptor bound uPA. ^{125}I PAI2 accumulated in tumour xenografts (approx. 1.5% of total injected dose) especially after repeat intravenous injections of ^{125}I -PAI2 without an accompanying increase in the major organs or in toxicity. In addition, tumour associated ^{125}I -PAI2 correlated with tumour mass. These results suggest that exogenously administered PAI2 efficiently targets uPA expressing tumours.

EXAMPLE 4**BREAST CANCER MODEL**

Two million human breast cancer cells [MDA-MB-231 (mycoplasma free)] in sterile phosphate buffered saline (PBS) were injected sub-cutaneously into the mammary fat pad of first pair breasts on both sides of 4-6 week old female nude mice.

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At 2, 7 and 14 days post cell inoculation, each mouse was injected s.c. at the site of tumour cell inoculation with either PBS (control) or varying doses of α -PAI2 in PBS (described as Bi-213-cDPTAa-PAI2 in figures titles; specific activity 3-4 μ Ci/10 μ L). The maximum volume injected at any one time was 100-120 μ l/site. Note that all mice were handled similarly and kept under similar feeding and housing conditions. The results are shown in figures 7, 8, 9 and 10.

The tumour volume was recorded for each mouse at various time points since inoculation of breast cancer cells (indicated on x-axis of figures as days after cell injection). The skin in area of inoculation was monitored for any signs of inflammation or damage - none were recorded. Also weight and over-all well-being was monitored from time of cell inoculation. These were all normal except for a small decrease in weight (~10%) immediately after alpha-PAI2 which was quickly recovered (data not shown). A similar phenomenon was seen with alpha-PAI2 treatment of mice without tumours in the alpha-PAI2 tolerance study (Figure 6 from Example 3).

The mice are monitored for another 2-3 weeks after which they are sacrificed for the purpose of performing histological analysis of tissues associated with the injection site, including axillary lymph node tissue for the presence of cancer cells.

Figures 7, 8, 9 and 10 show that significant reduction in tumor volume occurred with injection of radio-conjugate PAI-2. This was apparent with two doses of PAI-2, 25 Ci (Fig. 7) or 50 Ci (Fig. 8) injected 2 days after the inoculation of cancer cells into the mammary fat pad of the mouse. It was also apparent when the radio-conjugate PAI-2 was injected 7 days (Fig.9) or 14 days (Fig. 10) after the tumor cells.

These data clearly demonstrate the anti-tumor effectiveness of the radio-conjugated PAI-2

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Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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DATED this 23rd day of February, 2000

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By their Patent Attorneys
DAVIES COLLISON CAVE

Figure 1. Conjugated PAI2 forms a SDS-stable complex with uPA

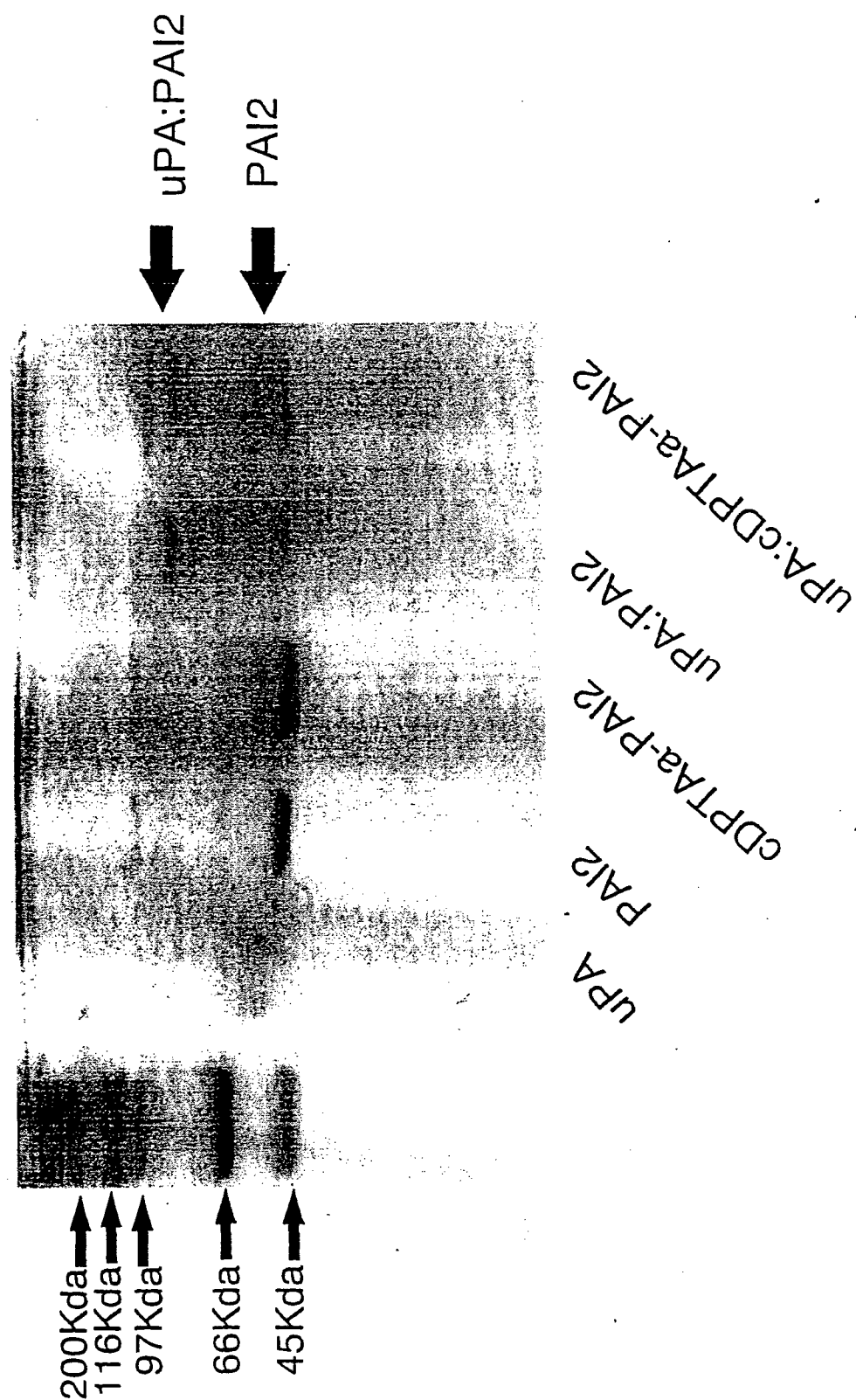


Figure 2 ITLC human serum stability results of PAI-2-cDTPAa and BSA-cDTPAa conjugates labelled with Bi-213

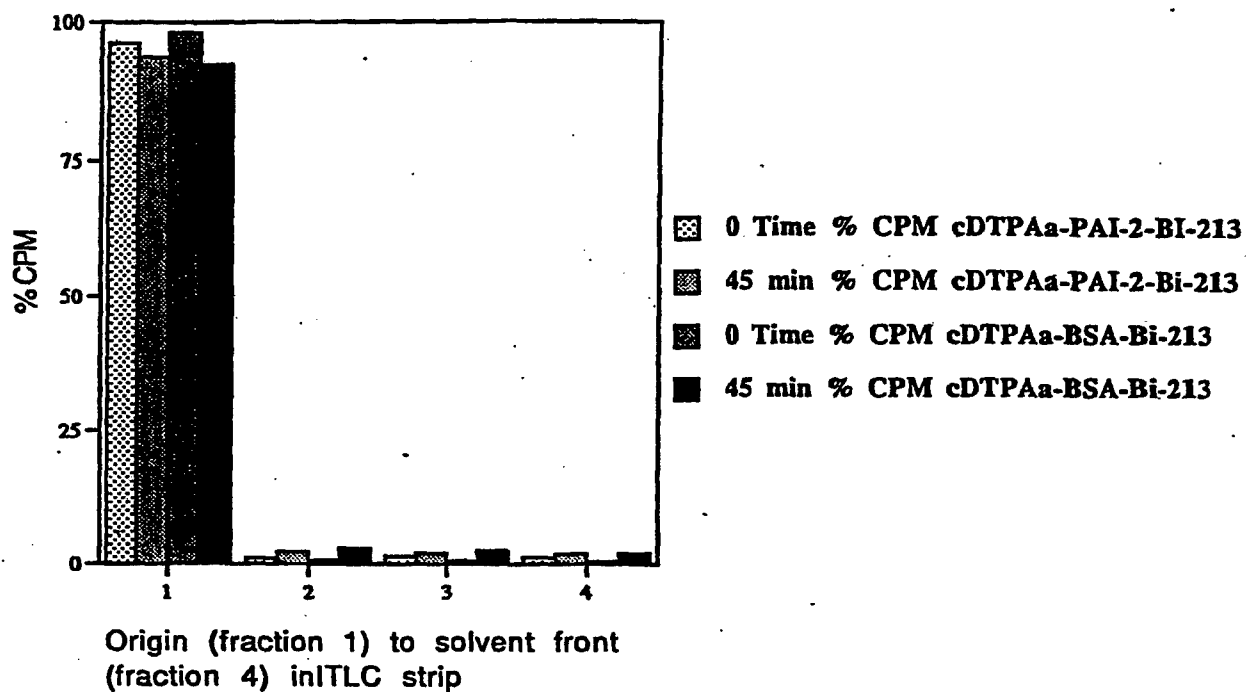


FIGURE 3

MDA-MB-231 cell survival

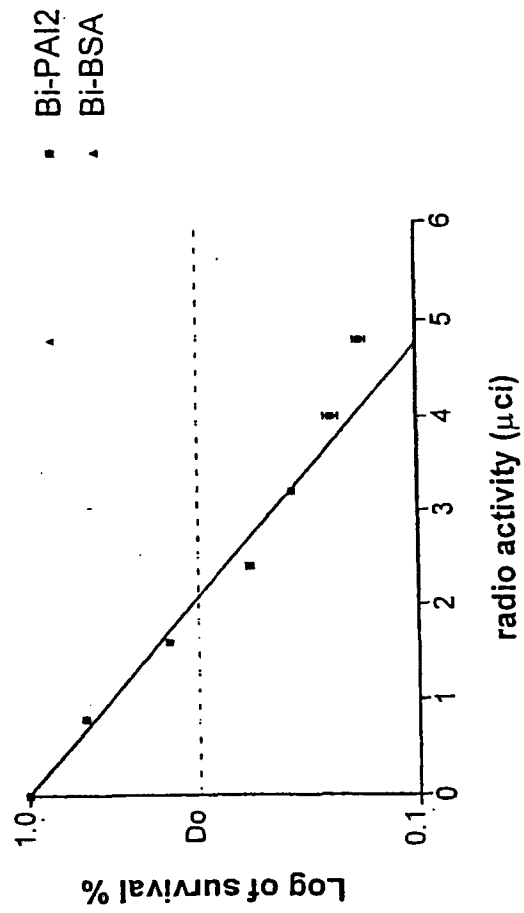


FIGURE 4

MCF-7 cell survival

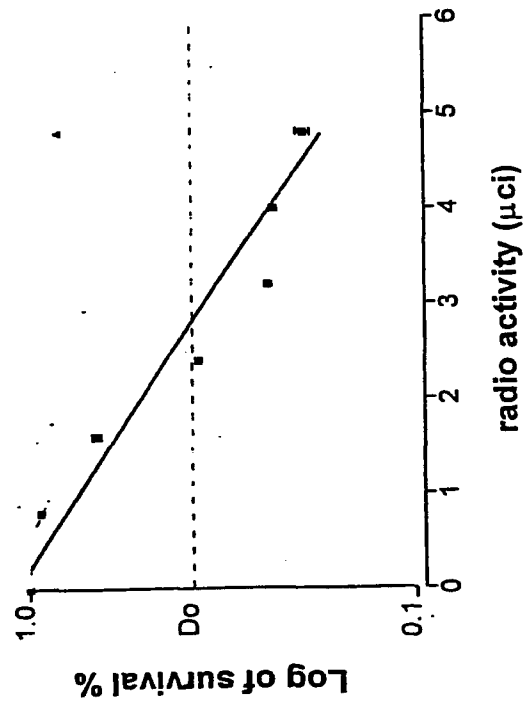
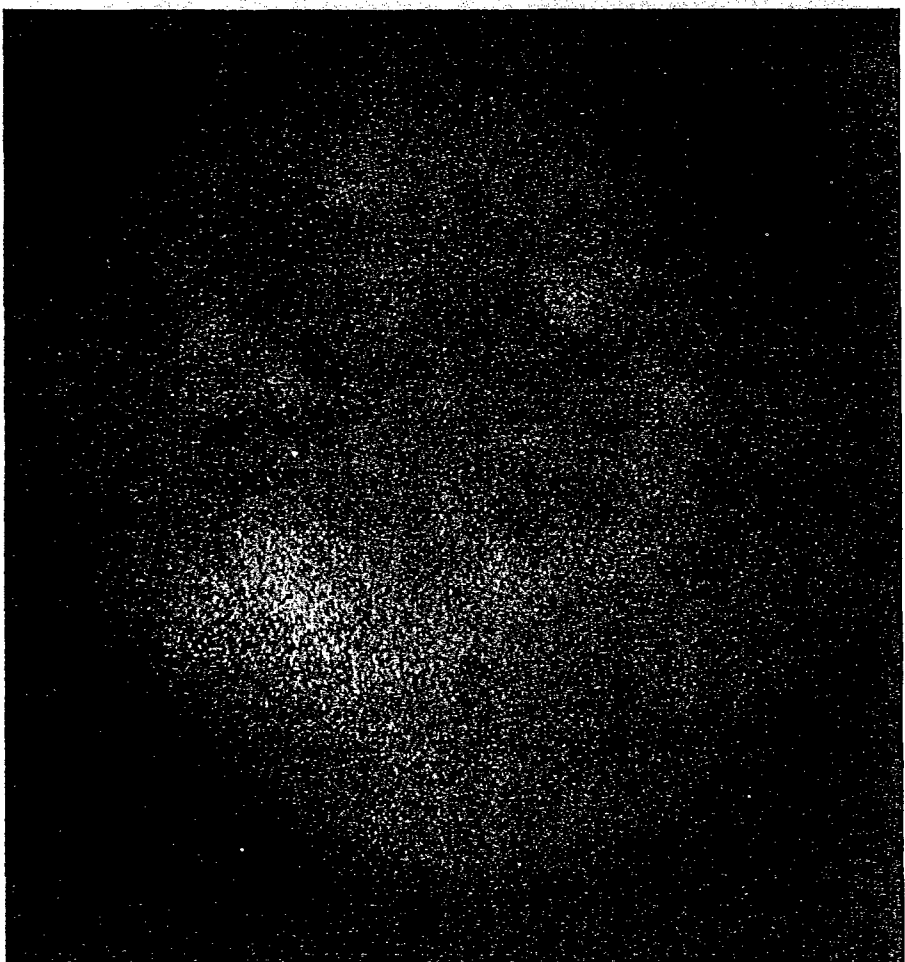
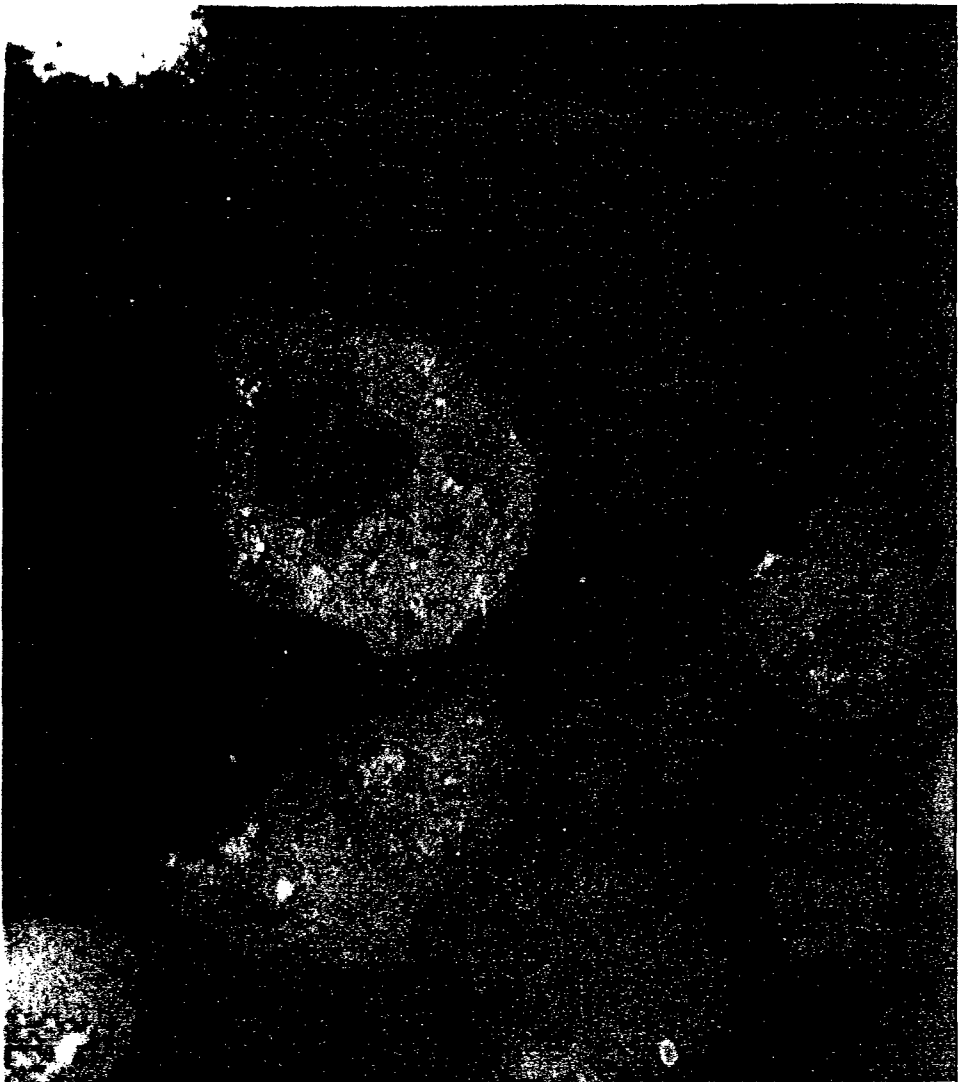


FIGURE 5

MDA-MB231 (GFP)



✓ GFP

■ PAI-2/TRITC

FIGURE 6

Alpha-PAI2 tolerance study in nude mice
(3 mCi/kg)

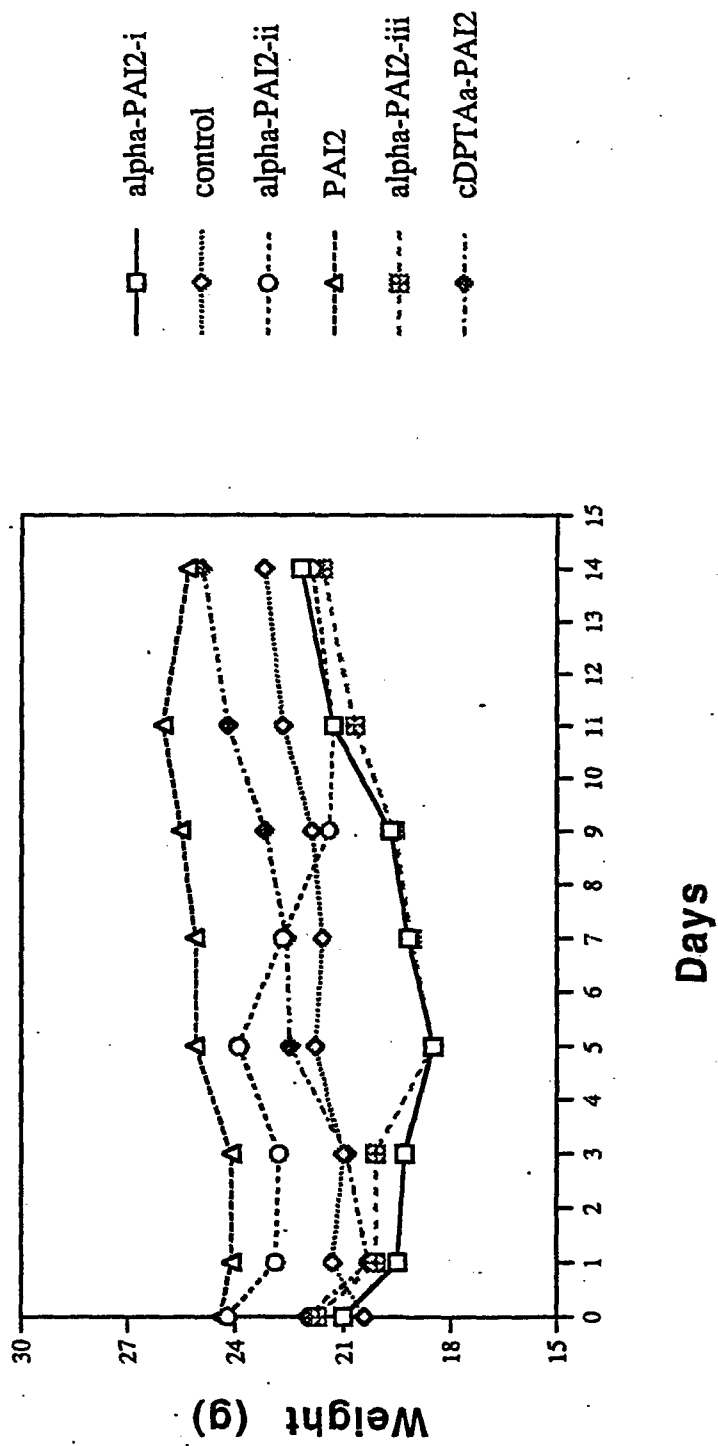


Figure 7.

TAT BREAST CANCER IN VIVO STUDY ON NOV. 1999 (Bi213- cDTPA-PAI2) Day2-25uci Group

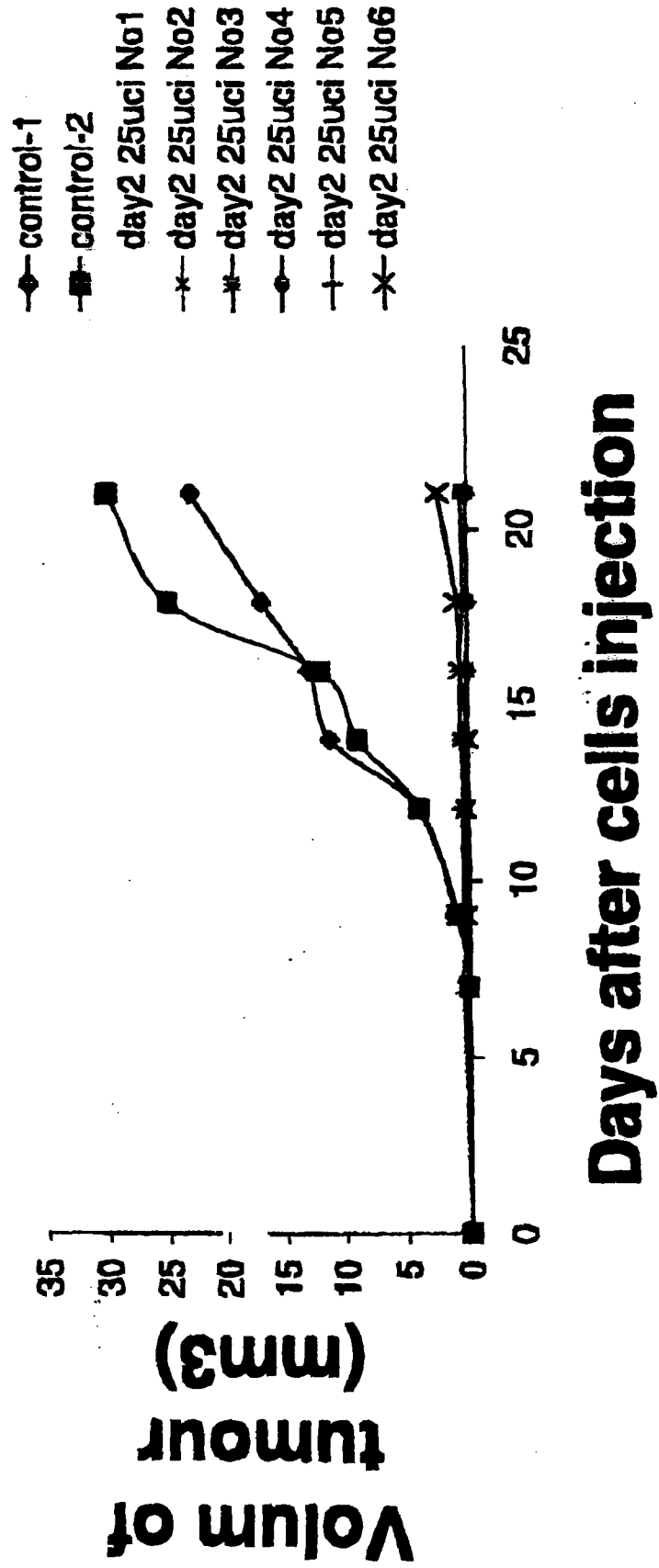
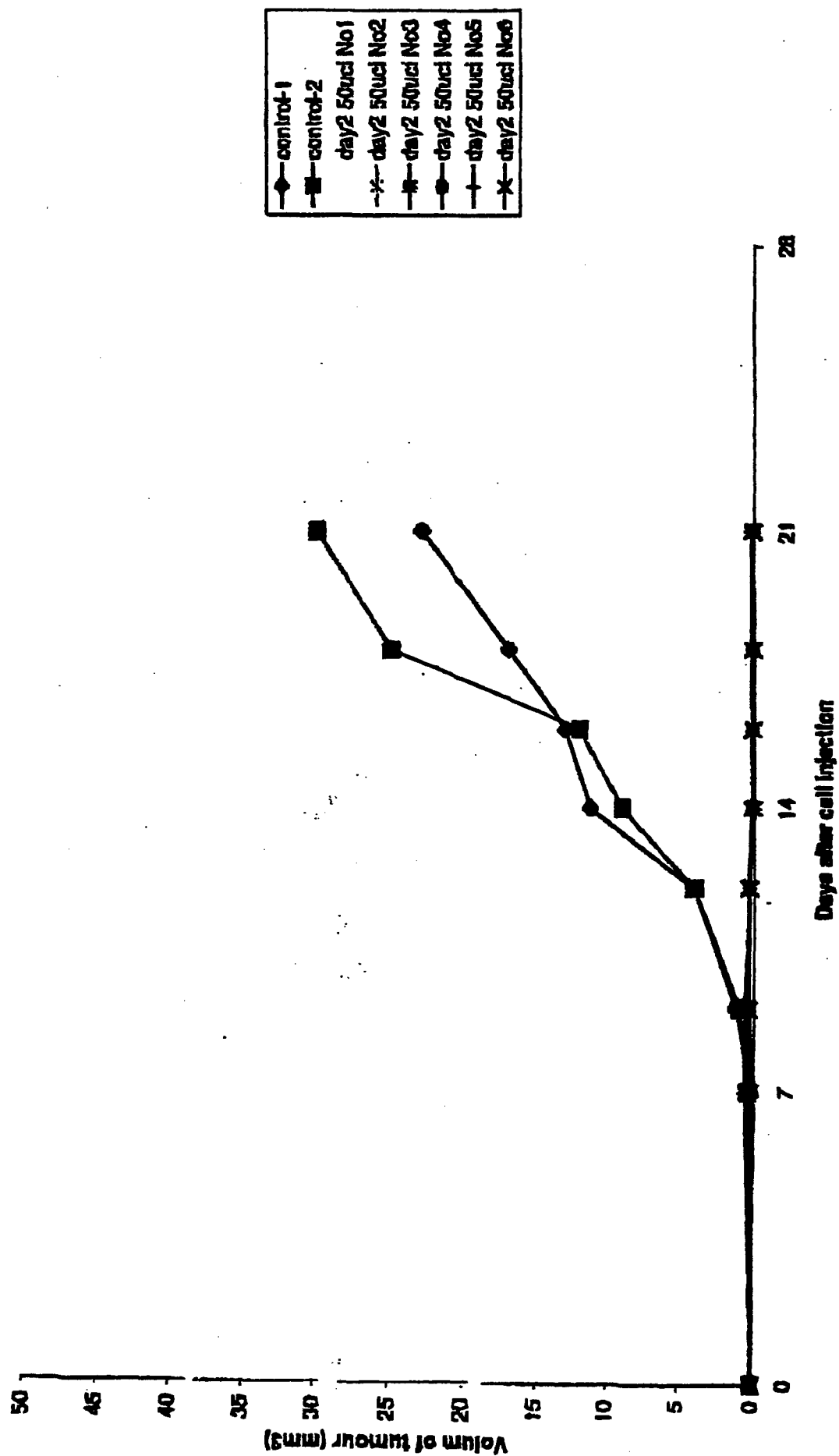


Figure 8.

TAT Breast Cancer In vivo Study (50 uci Bi-213-cDTPAs-PAI2 local inj. at day 2)



**TAT Breast Cancer in vivo Study
(25 μ ci BI-213-cDTPA-PA12 local inj. at day 7)**

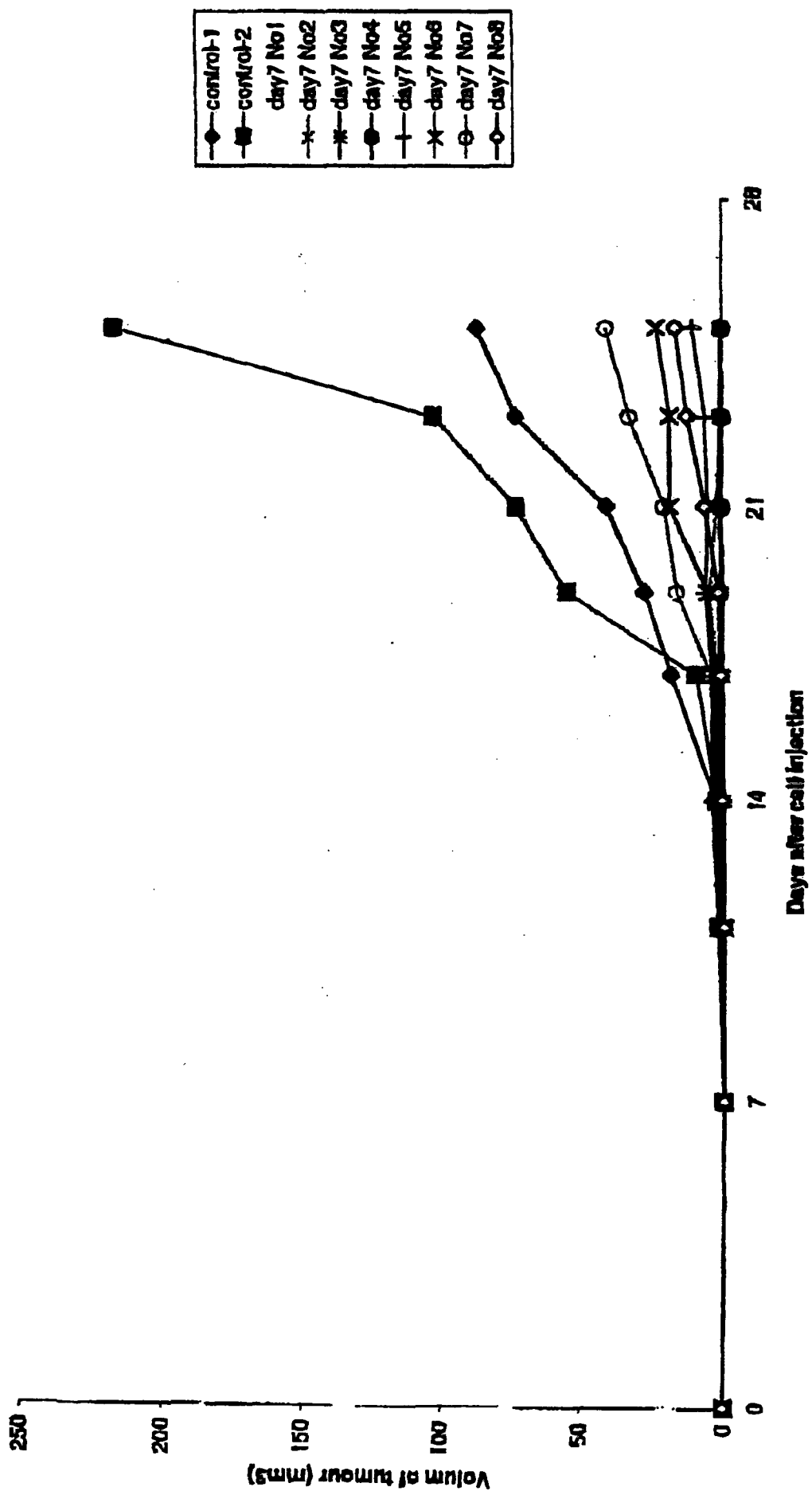
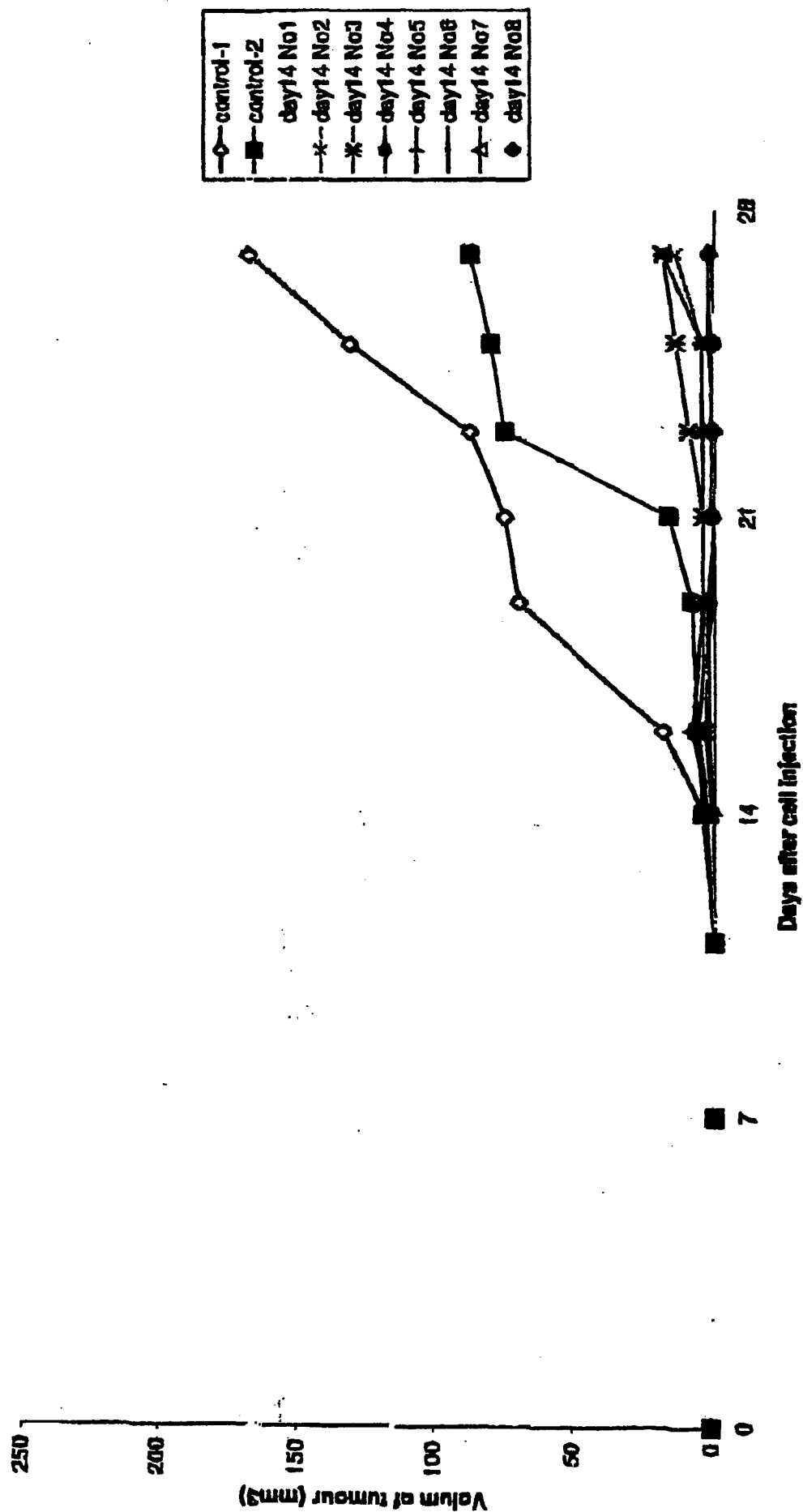


Figure 10.

TAT Breast Cancer in vivo Study
(25 uci Bi-213-cDTPAa-PAI2 local inj. at day 14)





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REPLY TO
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10 Jun 03

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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