Comparison of Radiohaloanalogues of Meta-Iodobenzylguanidine (MIBG) for a Combined Gene- and Targeted Radiotherapy Approach to Bladder Carcinoma

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Abstract: Targeted radiotherapy using radiolabelled meta-iodobenzylguanidine (MIBG) is a promising treatment option for bladder cancer, restricting the effects of radiotherapy to malignant cells thereby increasing efficacy and decreasing morbidity of radiotherapy. We investigated the efficacy of a combined gene therapy and targeted radiotherapy approach for bladder cancer using radiolabelled MIBG. The effectiveness of alternative radiohalogens and alternative preparations of radiolabelled MIBG for this therapeutic strategy were compared.

Bladder cancer cells, EJ138, were transfected with a gene encoding the noradrenaline transporter (NAT) under the control of a tumour specific telomerase promoter, enabling them to actively take up radiolabelled MIBG. This resulted in tumour-specific cell kill. Uptake and retention of radioactivity in cells transfected with the NAT gene were compared with that obtained in cells transfected with the sodium iodide symporter (NIS) gene. Substantially greater uptake and longer retention of radioactivity in NAT-transfected cells was observed. Carrier-added (c.a.) [^{131}I]MIBG, no-carrier added (n.c.a.) [^{131}I]MIBG, and [^{211}At]-labelled benzylguanidine (i.e. [^{211}At] meta-astatobenzylguanidine (MABG)) were compared with respect to efficiency of induction of cell kill. N.c.a[^{131}I]MIBG was more cytotoxic than c.a.[^{131}I]MIBG. However, the α-emitter [^{211}At]MABG was, by three orders of magnitude, more effective in causing tumour cell kill than the β-emitter [^{131}I]MIBG. We conclude that NAT gene transfer combined with the administration of n.c.a.[^{131}I]MIBG or [^{211}At]MABG, is a promising novel treatment approach for bladder cancer therapy.

Key Words: Bladder cancer, gene therapy, NAT, NIS, [^{131}I]MIBG, [^{211}At]MABG.

INTRODUCTION

Targeted tumour-specific radiotherapy, the selective irradiation of tumour cells with sparing of normal tissue, is achieved by the delivery specifically to malignant deposits, of cytotoxic radionuclides bound to tumour-seeking agents. A promising targeting agent is [^{131}I]meta-iodobenzylguanidine ([^{131}I]MIBG), a stable, non-immunogenic analogue of adrenergic neurone blockers. This β-emitter is used to treat neural crest tumours, such as phaeochromocytoma, neuroblastoma and paraganglioma [1-3]. These tumours express the membrane-bound noradrenaline transporter (NAT), enabling them to selectively concentrate [^{131}I]MIBG, resulting in specific irradiation of the target tumour cells. Treatment with radiolabelled MIBG is well tolerated in these patients, with relatively few side effects. Although cure generally is not yet achieved in patients treated with [^{131}I]MIBG as a single treatment agent, long-term remissions and good palliation have been obtained in patients with resistant disease. Another therapeutically valuable low molecular weight radiopharmaceutical is sodium radioiodide ($Na^{131}I$). $Na^{131}I$ is an effective treatment for disseminated thyroid cancers, which naturally express the sodium iodide symporter (NIS) and have an iodide organification pathway.

The recent characterisation of NAT and NIS genes [4, 5], encoding the transporters responsible for the active uptake of [^{131}I]MIBG and $Na^{131}I$, has raised the possibility of NAT and NIS gene transfer to enable targeting of a wide variety of tumour types with radiolabelled [^{131}I]MIBG and $Na^{131}I$ [6-14]. However, the absence of an iodide organification pathway in cells other than thyroid cells has the undesirable consequence of rapid iodide egress. Despite attempts to enhance iodide retention in cells after NIS gene transfer, efflux still represents the major limitation of this therapeutic strategy [15, 16]. In contrast, radioiodinated MIBG exhibits longer intracellular retention time.

Bladder cancer is a significant public health problem, causing, worldwide, more than 130,000 deaths annually. In the developed world, it is the fourth most common malignancy in males and tenth in females [17-19]. Ninety percent of bladder tumours are transitional cell carcinomas (TCC). Therapy of this malignancy is a significant challenge. Treatment of superficial disease is, often unsuccessfully, directed toward bladder preservation and toward the

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1573-4064/05 $50.00+.00

prevention of recurrence and progression to invasive disease [17, 20, 21]. The standard treatments of invasive TCC - radical cystectomy and radical external beam radiotherapy - are associated with high morbidity and mortality [17, 21-23]. While 5-year survival for patients with superficial disease is 70%, the 5-year survival for patients with muscle-invasive disease ranges from 16 to 59%, depending on the depth of invasion. Cystectomy tends to be the gold standard, but many patients are not suitable candidates for major surgery. Further, with an increasing concern about quality of life issues, such as bladder preservation, radiotherapy is becoming increasingly popular [24, 25]. Presently, the standard radiation regimen is a conventional dose and fractionation schedule resulting in a total dose of 60 to 66 Gy. The importance of total dose for survival is known but the dose that can be administered is limited by the response of normal tissue. 70% of patients experience acute self-limiting bladder and intestinal complications and up to 50% experience late complications in the form of bladder contracture, irritative voiding symptoms and haemorrhagic cystitis. The rate of severe acute bowel complications requiring surgery or at least 30 days of hospitalization is 2-17% [26-28].

New treatment modalities for bladder cancer are clearly required and a lot of research is now focusing on the potential of gene therapy [28-37]. A targeted tumour-specific radiotherapy for bladder cancer should avoid those side effects traditionally associated with external beam radiotherapy, by limiting radiation toxicity to neighbouring tissues and increasing the radiation dose to tumour cells, hence decreasing relapse rate. We have previously described such a targeted radiotherapy approach for bladder cancer using [^{131}I]MIBG [38]. The expression of the NAT transgene is limited to bladder cancer cells by placing its transcription under the control of tumour-specific telomerase gene regulatory elements.

In the present study, in order to optimize this targeted radiotherapy strategy, we compare NAT and NIS gene transfer, the effectiveness of high and low specific activity preparations of [^{131}I]MIBG and the efficacy of the β-particle emitter [^{131}I]MIBG with that of the α-particle emitter [^{211}At]MABG.

EXPERIMENTAL SECTION: MATERIALS AND METHODS

Plasmids

The bovine NAT cDNA was donated by Dr Michael Bruss and Prof. Heinz Bonisch (University of Bonn, Germany). Bovine NAT cDNA was used to allow discrimination between expression of exogenous and endogenous NAT in bladder cancer cell lines. Recombinant plasmids containing NAT cDNA, under control of either the universal viral CMV promoter or the RNA component of the telomerase promoter, were constructed as previously described [6, 8]. The telomerase promoter was provided by Prof. Nicol Keith (Cancer Research UK Beatson Laboratories, Glasgow, UK). The NAT gene fragment was subcloned into a promoterless pEGFP-1 plasmid (Clontech, BD Biosciences, Cowley, Oxford, UK) from which the EGFP gene had been removed. The promoter fragments were then subcloned into the multiple cloning site of the NAT-containing promoterless plasmid.

The human NIS cDNA, inserted into the EcoRI site of the pcDNA3 plasmid under the control of a CMV promoter, was provided by Dr S M Jhiang (Ohio State University, Columbus, OH, USA) [10].

In addition, as a transfection control plasmids, a pcDNA3 plasmid-only control, termed empty vector control [10]; and a negative control plasmid containing the NAT gene with no promoter element (pr- NAT) were used [8].

Target Cell Line and Culture Conditions

EJ138, a cell line derived from a human TCC of bladder, was cultured in MEM (Eagle), 25mm Hepes with Earle's Salt supplemented with foetal bovine serum (10%, v/v - Autogen Bioclear, Mile Elm Calne, Wiltshire, UK), L-glutamine (2mM), non-essential amino acids (0.1nM), fungizone (2ug/ml), and penicillin/streptomycin (100U/ml) at 37^{o}C with 5% CO_2.

Plasmid Transfection

Cells cultured in six-well plates were transfected using the Effectene transfection agent Kit (Qiagen Ltd. UK and Ireland) with plasmids containing either the NIS gene controlled by the CMV promoter (CMV/NIS), the NAT gene controlled by the CMV promoter (CMV/NAT) or the NAT gene controlled by the RNA component of the telomerase promoter (hTR/NAT). Further, as controls cells were transfected with the pcDNA3 plasmid-only control, termed empty vector control (EVC) and a negative control plasmid containing the NAT gene with no promoter element (pr-NAT).

After 24 h, cells were washed with phosphate buffered saline (PBS) and fresh medium was added. After 72 h, 0.5mg/ml of geneticin G418 sulphate (Gibco/Invitrogen Ltd.) was added to select for transfected cells. Transfectants were maintained in identical conditions to the parental cells, with the addition of geneticin G418 sulphate at each passage.

Radionuclide Uptake

The parental non-transfected EJ138 cell line and the transfectants expressing the NAT or NIS gene were seeded in 6-well plates prior to uptake studies until 70-80% confluent. To assess the presence of a functional NAT-transporter, radionuclide uptake was measured by incubating the parental cells and the NAT-transfected cells, in each well for 2 h with 7 kBq of [^{131}I]MIBG. Non-specific uptake was measured in controls treated with desmethylimipramine (DMI), an inhibitor of noradrenaline uptake [6, 39].

To evaluate NIS-transporter functionality, parental non-transfected EJ138 cells, the NIS-transfected cells and ECV-transfected cells were incubated for 1 h in a buffer containing 10 μm NaI and 7 kBq Na^{131}I. Non-specific uptake was measured in controls treated with perchlorate, an inhibitor of sodium iodide uptake [10, 39]. After incubation, cells were washed 3 times with ice-cold PBS. Radioactivity was then extracted with 10% (w/v) trichloroacetic acid. The activities of the extracts were measured in a γ-counter (Cobra II Auto-Gamma Counting System, Packard Instrument

Company, Meriden, CT, USA). Uptake was expressed as cpm per 10^5 cells. Specific uptake of radioactivity was calculated by subtracting values obtained in the presence of DMI or perchlorate from uninhibited uptake. Statistical analysis was performed using paired t-tests to determine whether differences in mean uptake between inhibited controls and non-inhibited transfectants were statistically significant.

Cellular Retention of Radioactivity

Cultures were treated as described for uptake studies and cell numbers were counted in parallel. After exposure of cells to [^{131}I]MIBG or Na^{131}I, the radioactive medium was replaced with fresh medium, which was later removed at various time points and radioactivity was measured in a γ-counter. The radioactivity in TCA extracts of the cells was also measured in a γ-counter. The activity associated with the medium indicated effluxed radioactivity. The counts derived from the TCA extracts provided an index of retained radioactivity. Data were expressed as cpm/10^5 cells.

Radiochemicals

Chemicals were purchased from Aldrich Chemical Company (Dorset, UK). HPLC grade solvents were obtained from Rathburn Chemicals (Peebleshire,UK). Carrier free sodium [^{131}I]iodide was purchased from Amersham Biosciences (Buckinghamshire UK). Therapy grade c.a.[^{131}I] MIBG (specific activity 1110 MBq/mg) was obtained from Amersham Health (Buckinghamshire,UK). C.a.[^{131}I]MIBG, of specific activity 45-65 MBq/mg, for determination of uptake capacity *in vitro* was obtained from Bristol Myers Squibb Pharmaceuticals Medical Imaging, (Chester, UK). N.c.a. [^{131}I]MIBG was synthesised by the electrophillic iododesilylation of the corresponding trimethylsilylbenzylguanidine analogue, as described previously [40]. N.c.a. [^{211}At]MABG was synthesised by astato-demetalation of tributylstannylbenzylamine as previously described [41].

Clonogenic Assay

To assess the toxicity of [^{131}I]MIBG, clonogenic assays were performed in spheroid models. Spheroids of EJ138 parental and transfected cells were grown by continuously stirring 3 x 10^6 cells in 90ml of medium in Techne stirrer flasks (Techne, Cambridge, UK) for 2-3 days until a size of approximately 300μm diameter was obtained.

Spheroids were transferred to 20ml universal containers, suspended in 1ml of medium containing the appropriate concentration of c.a.[^{131}I]MIBG, n.c.a.[^{131}I]MIBG, or [^{211}At] MABG, respectively, and incubated with agitation for 2 hours. Spheroids were washed twice with PBS before addition of 5ml of fresh medium and incubation at 37^{o}C in a 5% CO_2 atmosphere for 48 hours to allow radiation crossfire to occur [7, 42]. Spheroids were then washed twice with PBS, trypsinised, and manually disaggregated using a 5ml pipette. For each radioactivity concentration, three 25cm^2 flasks were seeded at 500 cells/flask and three at 750 cells/flask. The cells were then equilibrated with 5% CO_2 and incubated at 37^{o}C. After 9 days the resulting colonies were fixed and stained with 10% Gram's Crystal Violet solution (BDH Laboratories Supplies, Poole, BH, UK). Colonies of more than 50 cells were scored.

For statistical analysis of clonogenic assay data, two-sample t-tests were performed to determine the statistical significance of the differences in mean SF between EJ138 cells and transfectants expressing NAT.

RESULTS

Uptake of [^{131}I]MIBG and Na^{131}I

To assess the expression of transgenes in the transfectants, radionuclide uptake was measured in EJ138 cells transfected with the recombinant plasmids containing the NAT or NIS cDNA. Fig. (**1**) shows the active uptake capacity of the parental EJ138 cell line and the transfectants. There was negligible active accumulation of radioactivity in non-transfected EJ138 cells. In contrast, cells transfected with the NAT gene under the control of the ubiquitous CMV promoter or the hTR telomerase promoter exhibited a 25-fold or 36-fold enhancement, respectively, of active uptake of [^{131}I]MIBG relative to the same cells treated with the monoamine uptake inhibitor, desmethylimipramine (DMI). EJ138 cells transfected with the plasmid containing the bNAT cDNA with no promoter element (EJ138/pr- NAT) showed negligible active accumulation indicating that the bNAT transgene alone without promoter was in sufficient to allow expression of a functional NAT protein. Cells transfected with NIS under the control of the CMV promoter exhibited a 5-fold enhancement of active uptake of Na^{131}I relative to the same cells treated with the iodide uptake inhibitor, perchlorate. As with the non-transfected parental cells, cells transfected with empty control vector (EJ138-ECV) showed no significant active uptake of radioactivity. In cell lines transfected with NAT and NIS under the control of a promoter, the differences in uptake between the inhibited controls and the non-inhibited samples were statistically significant ($p<0.005$ for EJ138-CMV/NAT and EJ-CMV/NIS; $p<0.001$ for EJ-138hTR/NAT).

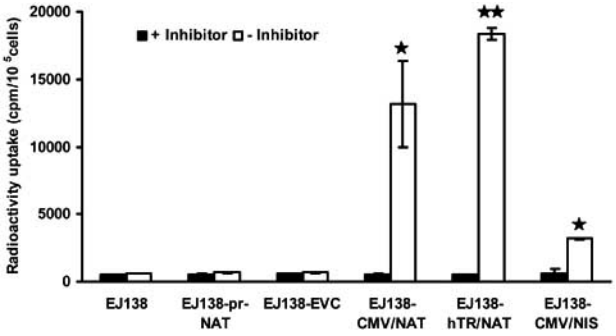

Fig. (1). Uptake of radioactivity in non-transfected EJ138 cells, EJ138 cells transfected with bNAT cDNA with no promoter element (EJ138/pr- NAT) and transfected with an empty control vector (EJ138- ECV) transfected and EJ138 cells transfected with the NAT or NIS gene under the control of the universal CMV promoter (EJ138-CMV/NAT or EJ138-CMV/NIS respectively), or the RNA component of the telomerase promoter (EJ138-hTR/NAT).
Experimental data represent means ± SD of three experiments performed in triplicate. The differences in uptake between the inhibited controls and the non-inhibited samples for EJ138-CMV/NAT, EJ138-hTR/NAT and EJ138-CMV/NIS are statistically significant (★ $p<0.005$, ★★$p<0.001$) .

These results demonstrate that transfection of both the NAT and NIS transgenes into bladder cancer cells resulted in expression of functional transporters.

Cellular Retention of Radioactivity

The rate of efflux of radioactivity after active uptake is presented in Fig. (**2**). Egress was faster from NIS-transfected cells than from NAT-transfected cells. The time for loss of half of the radioactivity from NAT-transfected cells was 9 h, and less than 5 minutes from NIS-transfected cells. The release of [^{131}I]MIBG from the NAT transfectants compares favourably with that previously observed in neuroblastoma cells: only 20% of [^{131}I]MIBG was retained after 3-4 hours [43]. Efflux of Na^{131}I from the NIS-transfected bladder cancer cells is similar to that reported for NIS-transfected glioma cells which lost 50% of accumulated Na^{131}I after 3 minutes [10].

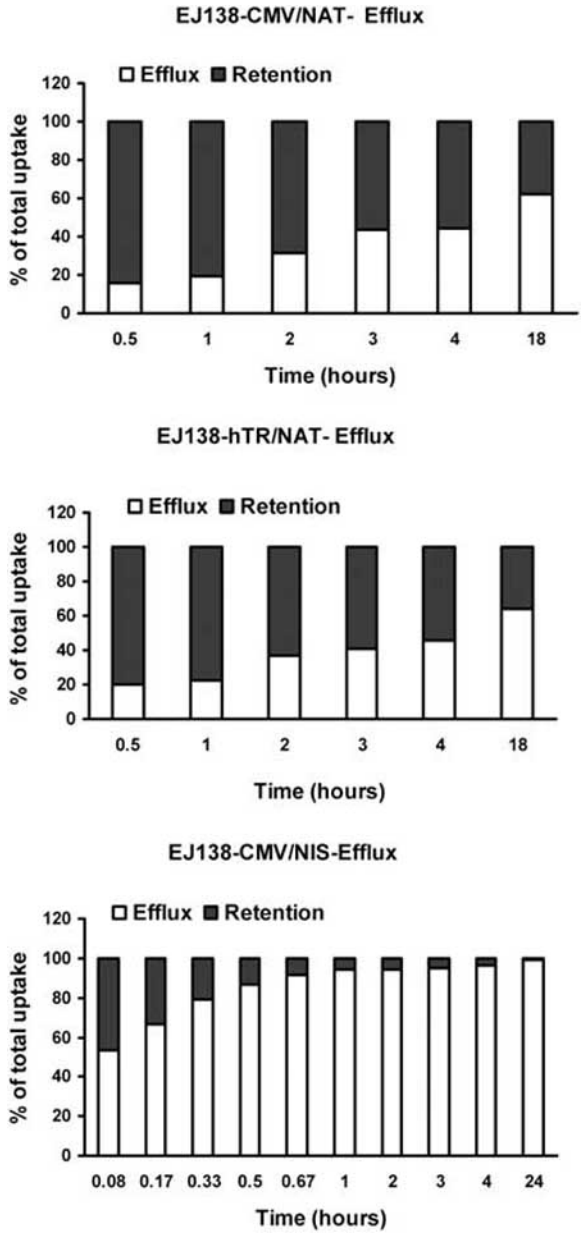

Fig. (2). Efflux and retention of radioactivity in EJ138 transfectants expressing NAT under the control of (a) the CMV promoter (EJ138-CMV/NAT) and (b) the hTR promoter (EJ138-hTR/NAT) and (c) NIS under the control of the CMV promoter (EJ138-CMV/NIS).

Dose-Dependent Toxicity of [^{131}I]MIBG to EJ138 Parental Cells and Transfectants Expressing NAT

Clonogenic assays were performed to determine whether uptake of [^{131}I]MIBG translated into dose-dependent cell kill. An [^{131}I]MIBG dose-dependent reduction in surviving fraction (SF) was observed for cells from disaggregated spheroids composed of NAT-transfected cells (Table (**1**)). After treatment with 10 MBq/ml c.a.[^{131}I]MIBG, the SF of clonogens from disaggregated spheroids was 0.543 for EJ138; 0.244 for EJ138-CMV/NAT; and 0.234 for EJ138-hTR/NAT. [^{131}I]MIBG uptake was reflected in cell kill in NAT-transfected EJ138 cells.

Clonogenic assays were also performed to compare the efficacy of c.a. and n.c.a.[^{131}I]MIBG. Greater cell kill was achieved with the n.c.a. preparation than with the c.a. preparation in the NAT transfected cells (Table (**1**)). SF values after treatment with 10 MBq/ml n.c.a.[^{131}I]MIBG were 0.518 for EJ138; 0.025 for EJ138-CMV/NAT; and 0.010 for EJ138-hTR/NAT.

Clonogenic cell kill after treatment with the α-emitter [^{211}At]MABG was greater than that induced by [^{131}I]-labelled MIBG, by approximately three orders of magnitude (Table **1**). Following treatment with 0.0222 MBq/ml of [^{211}At]MABG, SF was 0.589 for EJ138, whereas all EJ138-CMV/NAT and EJ138-hTR/NAT clonogens were sterilised.

The differences in SF between EJ138 cells and the transfectants (EJ138-CMV/NAT, and EJ138-hTR/NAT), at all treatment doses of radiolabelled [^{131}I]MIBG and [^{211}At] MABG, were statistically significant ($p<0.005$).

DISCUSSION

The combination of NAT gene transfer and radionuclide targeting using [^{131}I]MIBG is a promising radiotherapy approach for bladder cancer. An alternative scheme, involving radioiodide treatment after transfer of the NIS gene, is less attractive because the intracellular retention of [^{131}I]MIBG by NAT-transfectants was superior to that of Na^{131}I by NIS-transfectants. N.c.a[^{131}I]MIBG offers advantages over the conventional c.a.[^{131}I]MIBG preparation, and should therefore be the preparation of choice in future studies. The application of the α-emitting radiopharmaceutical [^{211}At]MABG could make this targeted radiotherapy approach even more tumour specific and toxic to malignant cells.

The experiments described involving uptake and retention of radionuclide show NAT gene transfer to give cells the benefit of longer intracellular retention of radionuclide than NIS gene transfer. NAT gene transfection also appeared to result in a greater increase in active uptake of radio-pharmaceutical than transfer of the NIS gene, but direct comparison of level of uptake between these transfectants

Table 1. Clonogenic Survival After Treatment with c.a.[^{131}I]MIBG, n.c.a.[^{131}I]MIBG or [^{211}At]MABG of EJ138 Parental Cells or Transfectants Expressing NAT Under the Control of the CMV Promoter (EJ138-CMV/NAT) or hTR Promoter (EJ138-hTR/NAT)

EJ138 untransfected:				
c.a.[^{131}I]MIBG (MBq//ml)	2.5	5.0	7.5	10
SF	1.024	1.049	0.622	0.543
(SD)	(0.126)	(0.094)	(0.080)	(0.174)
n.c.a.[^{131}I]MIBG (MBq//ml)	2.5	5.0	7.5	10
SF	0.978	0.913	0.616	0.518
(SD)	(0.126)	(0.114)	(0.070)	(0.074)
[^{211}At]MABG (kBq//ml)	2.8	7.4	14.8	22.2
SF	0.980	0.861	0.693	0.589
(SD)	(0.140)	(0.084)	(0.055)	(0.102)
EJ138-CMV/NAT:				
c.a.[^{131}I]MIBG (MBq//ml)	2.5	5.0	7.5	10
SF	0.657	0.506	0.441	0.244
(SD)	(0.097)	(0.078)	(0.037)	(0.046)
n.c.a.[^{131}I]MIBG (MBq//ml)	2.5	5.0	7.5	10
SF	0.558	0.252	0.111	0.025
(SD)	(0.039)	(0.014)	(0.020)	(0.004)
[^{211}At]MABG (kBq//ml)	2.8	7.4	14.8	22.2
SF	0.471	0.016	0.002	0.0
(SD)	(0.040)	(0.002)	(0.001)	(0.0)
EJ138-hTR/NAT:				
c.a.[^{131}I]MIBG (MBq//ml)	2.5	5.0	7.5	10
SF	0.649	0.524	0.402	0.234
(SD)	(0.047)	(0.024)	(0.045)	(0.035)
n.c.a.[^{131}I]MIBG (MBq//ml)	2.5	5.0	7.5	10
SF	0.463	0.090	0.040	0.010
(SD)	(0.066)	(0.008)	(0.004)	(0.001)
[^{211}At]MABG (kBq//ml)	2.8	7.4	14.8	22.2
SF	0.453	0.014	0.001	0.0
(SD)	(0.063)	(0.003)	(0.0)	(0.0)

Data represent means ± SD.

expressing different genes is difficult, since it does not take compounding factors such as transfection efficiency and average number of CMV/NIS and CMV/NAT molecules per cell into account.

Restriction of the expression of the NAT transgene to malignant cells should be achievable by placing its transcription under the control of a tumour-specific telomerase promoter. Telomerase is up-regulated in malignant cells, and several studies have shown that telomerase promoters are suitable for tumour-specific expression of transgenes [44-48]. Whilst some non-cancerous tissue, such as male germ cells, lymphocytes and some stem cell populations express telomerase, there is a clear differential in expression levels between TCC and normal tissue [46, 49, 50]. Moreover, the option of intravesical administration of transgenes and radiopharmaceutical would be expected to minimise side effects to telomerase-expressing stem cells elsewhere in the body. Concerning the bladder itself, ninety two percent of TCC bladder tumours at all stages are telomerase-positive [49-55]. Low level telomerase expression was reported in normal bladder mucosa only under conditions of dysplasia and severe inflammation [49, 52, 54] accounting for a large differential in telomerase expression between normal bladder and tumour. These factors render the telomerase promoters suitable candidates for bladder cancer specific gene expression [38].

Targeted radiotherapy of tumours is achieved by the delivery, specifically to malignant deposits, of cytotoxic radionuclides bound to tumour-seeking agents. The advent of monoclonal antibody technology was accompanied by the prospect that all tumours could be targeted by exploiting the cell surface antigens on malignant cells. However, clinical application of these radiolabelled macromolecules have generally been unsatisfactory due to low tumour-specificity of targeted epitopes, limited penetration into tumours and the provocation of anti-mouse immunoglobulin responses. Some investigators have assessed intravesical administration of radiolabelled tumour-associated monoclonal antibodies as a means of preventing progression of superficial bladder cancer [56-58]. However, non-immunogenic low molecular weight radiopharmaceuticals with good penetrative properties and higher uptake in tumours, such as MIBG may offer several advantages, especially if combined with the transfer of a transporter gene. This approach may result in increased tumour retention of cytotoxic radionuclides; increased tumour specificity by virtue of telomerase activity in tumours; and avoidance of an anti-mouse antibody response.

The contained bladder space, the ease of intravesical administration, and the possibility of adenovirus-mediated gene transfer into urothelium [33, 34], indicate that gene therapy is a feasible option for TCC bladder. Several factors have limited the efficiency of adenovirus-mediated gene transfer. However, recently reported refinements to viral administration may overcome low efficiency of transduction. Pre-treatment with transduction-enhancing agents may improve adenovirus-mediated gene transfer into the urothelium across the protective glycosaminoglycan layer which otherwise acts as a non-specific antiadherence barrier [32, 59-62]. Furthermore, differentiation-inducing agents are capable of up-regulating coxsackie adenovirus receptor expression by bladder cancer cells thereby increasing the efficiency of adenoviral gene transfer [63, 64]. Studies performed in patients pre-cystectomy with adenovirus-mediated gene transfer by intravesical vector installation showed a uniform vector penetration throughout the urothelium as well as into submucosal tumour tissue in 7 out of 8 patients [33]. The added benefit of radiological bystander effects to a gene therapy approach could result in the control of malignant growth in the muscular layer. Moreover, local immune responses in the bladder, secondary to the adenovirus installation, are expected to enhance anti-tumour effect [34].

C.a.[^{131}I]MIBG contains a high amount of non-radiolabelled MIBG. The ratio of radioactive MIBG to non-radioactive MIBG molecules in commercially available c.a.[^{131}I]MIBG is only approximately 1:2000. In contrast, n.c.a.[^{131}I]MIBG, which is synthesised by iododesilylation of meta-trimethylsilylbenzylguanidine [40], contains for a given radioactivity only a maximum of 0.05% of the molar amount of MIBG compared [^{131}I]MIBG solutions prepared by iodide exchange [65]. MIBG uptake in cells expressing the NAT occurs by a high affinity, saturable and active mechanism and to a much lesser extent by passive diffusion (common to all cells). Non-radioactive MIBG competes with radiolabelled MIBG for the NAT. Further some side effects in the form of nausea and vomiting, experienced with current [^{131}I]MIBG therapy in neuroendocrine tumours, are due to the excess of unlabelled MIBG. In the current study, greater cell kill was achieved for a given radioactivity concentration in the form of n.c.a.[^{131}I]MIBG compared to c.a.[^{131}I]MIBG. Previous studies have also demonstrated enhanced cell kill in NAT expressing cells utilising the n.c.a. preparation [66-68]. We therefore propose that for therapeutic *in vivo* experiments or trials the n.c.a. preparation of [^{131}I]MIBG should be used.

In the present study, clonogenic assays were performed in three-dimensional spheroids rather than monolayers. Previously it has been shown that greater cell kill was achieved, for the same dose of [^{131}I]MIBG, in three-dimensional spheroids compared to monolayers, demonstrating the existence of collateral cell kill [38]. This is of great importance to the success of gene therapy, since gene transmission is an inefficient process. That is, 100% transfection efficiency cannot be achieved using available methodology [7, 11, 42]. Therefore is it necessary to delineate the mechanism of induction of radiation-induced bystander effects in order to maximise the effectiveness of gene therapy used in conjunction with targeted radionuclide treatment [7, 69, 70]. Two types of bystander effect have been identified: namely a physical effect in the form of decay particle, cross-fire irradiation from targeted cells to neighbouring, untargeted cells, observed mainly in conjunction with low linear energy transfer (LET) β-and γ-emitters; and transmissible biological effects resulting from the radiation insult. The latter phenomenon is more pronounced following high LET radiation, [71-73].

For the MIBG targeted radiotherapy of neuroectodermal tumours, the radionuclide conventionally used is ^{131}I. However, tumours of sub-millimetre dimensions are sub-optimal targets for treatment with ^{131}I β-particles whose

mean range is about 800μm [74]. In addition to underdosing of small tumour deposits, long range β-emissions may damage surrounding normal tissues [39, 75, 76]. Due to their short path length, radionuclides that decay by the emission of α–particles, such as the heavy halogen astatine-211 (^{211}At), offer the prospect of combining cell-specific molecular targeting with radiation having a range in tissue of only 50 to 80 μm [77, 78]. Moreover, α–particles are much more radiotoxic than β-emitting radionuclides and their cytocidal efficiency is independent of cell cycle status and oxygen concentration. Recently, clinical trials involving tumour-antibodies labelled with α–particle-emitting radionuclides have commenced [77, 79]. The combination of β-and α-emitters, in the form of [^{131}I]MIBG and [^{211}At]meta-astatobenzylguanidine ([^{211}At]MABG), could be an option for the treatment of bladder cancer, further increasing the efficiency and specificity of this targeted radiotherapy strategy.

This novel scheme combining gene therapy and targeted radiotherapy could play a part in the control of superficial TCC in order to prevent recurrence or progression. It may also be used in the treatment of residual malignant deposits after transurethral resection of bladder tumour. With respect to invasive TCC, this tumour-specific radiotherapy strategy could provide an effective, alternative radiation treatment to external beam radiotherapy. Furthermore, this treatment could help in the management of inaccessible upper tract TCC in the ureter or renal pelvis.

This new treatment strategy, combining a novel gene therapy approach with a well-established treatment option for bladder cancer – radiotherapy - might render radiation treatment more effective and more readily tolerated. The large differential in telomerase expression between normal tissue and bladder tumour, and the possibility of intravesical administration of treatment make bladder cancer an attractive candidate for this therapeutic scheme.

ACKNOWLEDGEMENTS

The authors' work is supported by the British Urological Foundation (BUF/ Sanofi-Synthelabo Scholarship 2001/ 2002); the Royal College of Physicians and Surgeons of Glasgow (Ian Sunter Charitable Trust Research Fellowship 2003); the Urology Cancer Research Fund 1122, Gartnavel General Hospital, Glasgow; Cancer Research UK; Glasgow University; the Department of Health and the NIH.

ABBREVIATIONS

c.a. = Carrier added

DMI = Desmethylimipramine

hTR = RNA component of telomerase

LET = Linear energy transfer

MABG = Meta-astatobenzylguanidine

MIBG = Meta-iodobenzylguanidine

NAT = Noradrenaline transporter

n.c.a. = No-carrier added

NIS = Sodium iodide symporte

PBS = Phosphate buffered salin

TCC = Transitional cell carcinoma

REFERENCES

[1] Gaze, M.N.; Wheldon, T.E. *Eur. J. Cancer*, **1996**, *32A*(1), 93.
[2] Troncone, L.; Rufini, V. *Anticancer Res.*, **1997**, *17*(3B), 1823.
[3] Mukherjee, J.J.; Kaltsas, G.A.; Islam, N; Plowman, P.N.; Foley, R;. Hikmat, J.; Britton, K.E.; Jenkins, P.J.; Chew, S.L.; Monson, J.P.; Besser, G.M.; Grossman, A.B. *Clin. Endocrinol. (Oxf.)*, **2001**, *55*(1), 47.
[4] Pacholczyk, T.; Blakely, R.D.; Amara, S.G. *Nature*, **1991**, *350*, 350.
[5] Dai, G.; Levy, O.; Carrasco, N. *Nature*, **1996**, *379*, 458.
[6] Boyd, M.; Cunningham, S.H.; Brown, M.M.; Mairs, R.J.; Wheldon, T.E. *Gene Ther*., **1999**, *6*, 1147.
[7] Boyd, M.; Mairs, S.C.; Stevenson, K.; Livingstone, A.; McCluskey A.G.; Ross, S.C.; Mairs, R.J. *J. Gene Med.*, **2002**, *4*, 1.
[8] Boyd, M.; Mairs, R.J.; Mairs, S.C.; Wilson, L.E.; Livingstone, A.; Cunningham, S.H.; McCluskey, A.; Brown, M.M.; Quigg, M.; Keith, N. *Oncogene*, **2001**, *20*, 7804.
[9] Boland, A.; Ricard, M.; Opolon, P.; Bidart, J.M.; Yeh, P.; Filetti, S.; Schlumberger, M.; Perricaudet, M. *Cancer Res.*, **2000**, *60*, 3484.
[10] Carlin, S.; Cunningham, S.H.; Boyd, M.; McCluskey, A.G.; Mairs, R.J. *Canc. Gene Ther.*, **2000**, *7*, 1529.
[11] Mairs R.J.; Cunningham S.H.; Boyd M.; Carlin S. *Cur. Pharm. Des.*, **2000**, *6*, 1419.
[12] Spitzweg, C.; O'Connor, M.K.; Bergert, E.R.; Tindall, D.J.; Young, C.Y.F.; Morris, J.C. *Cancer Res.*, **2000**, *60*, 6526.
[13] Spitzweg, C.; Deitz, A.B.; O'Connor, M.K.; Bergert, E.R.; Tindall,D.J.; Young, C.Y.F.; Morris, J.C. *Gene Therapy*, **2001**, *8*, 1524.
[14] Fullerton, N.E.; Boyd, M.; Mairs, R.J.; Keith, W.N.; Alderwish, O.; Brown, M.M.; Livingstone, A.; Kirk, D. *Prostate Cancer Prostatic Dis.*, **2004**, *7*(4), 355.
[15] Huang, M.; Batra, R.K.; Kogai, T.; Lin, Y.Q.; Hershman, J.M.; Lichtenstein, A.; Sharma, S.; Zhu, L.X.; Brent, G.A.; Dubinett, S.M. *Cancer Gene Therapy,* **2001**, *8*, 612.
[16] Boland, A.; Magnon, C.; Filetti, S.; Bidart, J.M.; Schlumberger, M.; Yeh, P.; Perricaudet, M. *Thyroid*, **2002**, *12*, 19.
[17] Pashos, C.L.; Botteman, M.F.; Laskin, B.L.; Redaelli, A. *Cancer Practice*, **2002**, *10*(6), 311.
[18] Borden, L.S. Jr.; Clark, P.E.; Hall, M.C. *Curr. Opin. Oncol.*, **2003**, *15*(3), 227.
[19] Cancer Research UK. In *Scientific Yearbook 2003/2004*; Cancer Research UK, Ed.; **2004**, pp 8-10.
[20] Chopin, D.K.; Gattegno, B. *Eur. Urol.*, **2002**, *42*, 533.
[21] Oosterlinck, W.; Lobel, B.; Kakse, G.; Malmstroem, P.-U.; Stoeckle, M.; Sternberg, C. *Eur. Urol.*, **2002**, *41*, 105.
[22] Clark, P.E.; Klein, E.A. Curr. *Opin. Urol.*, **1999**, 9(5), 413.
[23] Petrovich, Z.; Jozsef, G.; Brady, L.W. *Am. J. Clin. Oncol.*, **2001**, *24*(1), 1.
[24] Gospodarowicz, M. *J. Clin. Oncol.*, **2002**, *20*(14), 3048
[25] Kuczyk, M.; Turkeri, L.; Hammerer, P.; Ravery, V. *Eur. Urol.*, **2003**, *44*, 57-64.
[26] Marks, L.B.; Carroll, P.R.; Dugan, T.C.; Anscher, M.S. *Int. J. Radiation Oncology Biol. Phys.*, **1995**, *31*(5), 1257.
[27] Sengelov, L.; von der Maase, H. *Radiother. Oncol.*, **1999**, *52*(1), 1.
[28] Lopez-Beltran, A.; Luque, R.J.; Mazzucchelli, R.; Scarpell, M.; Montironi, R. *J. Clin. Pathol.*, **2002**, *55*, 641.
[29] Harimoto, K.; Sugimura, K.; Lee, C.R.; Kuratsukuri, K.; Kishimoto, T. *Br. J. Urol. International,* **1998**, *81*(6), 870.
[30] Malmstroem, P.-U. *Curr. Opin. Urol.*, **2000**, *10*, 447.
[31] Duggan, B.J.; Kelly, J.D.; Keane, P.F.; Johnston, S.R.; Williamson, K.E. *J. Urol*., **2001**, *165*, 946.
[32] Shimizu, H.; Akasaka, S.; Suzuki, S.; Akimoto, M.; Shimada, T. *Urology*, **2001**, *57*(30), 579.
[33] Kuball, J.; Wen, S.F.; Leissner, J.; Atkins, D.; Meinhardt, P.; Quijano, E.; Engler, H.; Hutchins, B.; Maneval, D.C.; Grace, M.J.; Fritz, M.A.; Stoerkel, S.; Thueroff, J.W.; Huber, C.; Schuler, M. *J. Clin. Oncol.*, **2002**, *20*, 957.
[34] Pagliaro, L.C.; Keyhani, A.; Williams, D.; Woods, D.; Liu, B.; Perrotte, P.; Slaton, J.W.; Merritt, J.A.; Grossman, H.B.; Dinney, C.P. *J. Clin. Oncol.*, **2003**, *21*(12), 2247.

[35] Sweeney, P.; Karashima, T.; Ishikura, H.; Wiehle, S.; Yamashita, M.; Benedict, W.F.; Cristiano, R.J.; Dinney, C.P.N. *Cancer Res.*, **2003**, *63*, 4017.
[36] Zhang, X.; Multani, A.S.; Zhou, J.-H.; Shay, J.W.; McConkey, D.; Dong, L.; Kim, C.-S.; Rosser, C.J.; Pathak, S.; Benedict, W.F. *Cancer Res.*, **2003**, *63*, 760.
[37] Lee ,C.-F.; Chang, S.-Y.; Hsieh, D.-S.; Yu, D.-S. *Cancer Gene Ther.*, **2004**, *11*, 194.
[38] Fullerton, N.E.; Mairs, R.J.; Kirk, D.; Keith, W.N.; Carruthers, R.; McCluskey, A.G.; Brown, M.; Wilson, L.; Boyd, M. *Eur. Urol.*, **2005**, *47*, 250.
[39] Cunningham, S.H.; Mairs, R.J.; Wheldon, T.E.; Welsh, P.C.; Vaidyanathan, G.; Zalutsky, M.R. *Br. J. Cancer*, **1998**, *77*(12), 2061.
[40] Vaidyanathan, G.; Zalutsky, M.R. Appl. Radiat. Isot. (Int. J. Radiat. Appl. Instrum. Part A), **1993**, *44*, 621.
[41] Vaidyanathan, G.; Zalutsky, M.R. *Bioconjugate Chem.*, **1992**, *3*, 499.
[42] Boyd, M.; Mairs, R.J.; Cunningham, S.H.; Mairs, S.C.; Stevenson, K.; McCluskey, A.G.; Carlin, S.; Brown, M.M.; Livingstone, A.; Wilson, L.; Wheldon, T.E. *J. Gene Med.*, **2001**, *3*, 165.
[43] Mairs, R.J.; Gaze, M.N.; Barrett, A. *Br. J. Cancer*, **1991**, *64*, 293.
[44] Zhao, J.Q.; Hoare, S.F.; McFarlane, R.; Muir, S;. Parkinson, E.K.; Black, D.M.; Keith, W.N. *Oncogene*, **1998**, *16*(10), 1345.
[45] Abdul-Ghani, R.; Ohana, P.; Matouk, I.; Ayesh, S.; Ayesh, B.; Laster, M.; Bibi, O.; Giladi, H.; Molnar-Kimber, K.; Sughayer, M.A.; de Groot, N.; Hochberg, A. *Mol. Ther.,* **2000**, *2*(6), 539.
[46] Plumb, J.A.; Bisland, A.; Kakani, R.; Zhao, J.; Glasspool, R.M.; Knox, R.J.; Evans ,T.R.J.; Keith, W.N. *Oncogene*, **2001**, *20*, 7797.
[47] Gu, J.; Fang, B. *Cancer Biol. Ther.*, **2003**, *2*(4 Suppl. 1), S64.
[48] Boyd, M.; Mairs, R.J.; Keith, W.N.; Ross, S.C.; Welsh, P.; Akabani, G.; Owens, J.; Vaidyanathan, G.; Carruthers, R.; Dorrens, J.; Zalutsky, M.R. *J. Gene Med.*, **2004**, *6*(8), 937.
[49] Keith, W.N.; Evans, T.R.J.; Glasspool, R.M. *J. Pathol.*, **2001**, *195*, 404.
[50] Melissourgos, N.; Kastrinakis, N.G.; Davilas, I.; Foukas, P.; Farmakis, A.; Lykourinas, M. *Urology*, **2003**, *62*, 362.
[51] Kim, N.W.; Piatyszek, M.A.; Prowse, K.R.; Harley, C.B.; West, M.D.; Ho, P.L.; Coviello, G.M.; Wright, W.E.; Weinrich, S.L.; Shay, J.W. *Science*, **1994**, *266*, 2011.
[52] Shay, J.W.; Bacchetti, S. *Eur. J. Cancer*, **1997**, *33*(5), 787.
[53] Liu, B.C.; Loughlin, K.R. *Urol. Clin. North Am.*, **2000**, *27*(1), 115.
[54] Orlando, C.; Gelmini, S.; Selli, C.; Pazzagli, M. *J. Urol.*, **2001**, *166*(2), 666.
[55] Mueller, M. *Oncogene*, **2002**, *21*(4), 650.
[56] Hughes, O.D.M.; Bishop, M.C.; Perkins, A.C.; Frier, M.; Price, M.R.; Denton, G., Smith, A.; Rutherford, R. *Eur. J. Nucl. Med.*, **1997**, *24*, 439.
[57] Hughes, O.D.M.; Bishop, M.C.; Perkins, A.C.; Wastie, M.L.; Denton, G.; Price, M.R.; Frier, M.; Denley, H.; Rutherford, R.; Schubiger, P.A. *J. Clin. Oncol.*, **2000**, *18*, 363.
[58] Syrigos, K.N.; Khawaja, M.; Krausz, T.; Williams, G.; Epenetos, A. *Acta Oncologica*, **1999**, *38*(3), 379.
[59] Engler, H.; Anderson, S.C.; Machemer, T.R.; Philopena, J.M.; Connor, R.J.; Wen, S.-F.; Maneval, C. *Urology*, **1999**, *53*(5), 1049.
[60] Connor, R.J.; Engler, H.; Machemer, T.; Philopena, J.M.; Horn, M.T.; Sutjipto, S.; Maneval, D.C.; Youngster, S.; Chan, T.-M.; Bausch, J.; McAuliffe, J.P.; Hindsgaul. O.; Nagabhushan, T.L. *Gene Ther.*, **2001**, *8*, 41.
[61] Yamashita, M.; Rosser, C.J.; Zhou, J.-H.; Zhang, X.-Q.; Connor, R.J.; Engler, H.; Maneval, D.C.; Karashima, T.; Czerniak, B.A.; Dinney, C.P.N.; Benedict, W.F. *Cancer Gene Ther.*, **2002**, *9*, 687.
[62] Ramesh, N.; Memarzadeh, B.; Ge, Y.; Fre,y D;. VanRoey, M; Rojas, V.; Yu, D.-C. *Mol. Ther.*, **2004**, *10*(4), 697.
[63] Okegawa, T.; Pong, R.-C.; Li, Y.; Bergelson, J.M.; Sagalowsky, A.I.; Hsieh, J.-T. *Cancer Res.*, **2001**, *61*, 6592.
[64] Sachs, M.D.; Ramamurthy, M.; van der Poel, H.; Wickham, T.J.; Lamfers, M.; Gerrirtson, W.; Chowdhury, W.; Li Y.; Schoenberg, M.P.; Rodriguez, R. *Cancer Gene Ther.*, **2004**, *11*, 477.
[65] Mairs, R.J.; Gaze, M.N.; Watson, D.G.; Skellern, G.G.; Constable, P.; McKellar, K; Owens, J.; Vaidyanathan, G.; Zalutsy, M.R. *Nucl. Med. Commun.*, **1994**, *15*, 268.
[66] Mairs, R.J.; Russel, J.; Cunningham, S.; O'Donoghue, J.A.; Gaze, M.N.; Owen, J.; Vaidyanathan, G.; Zalutsky, M.R. *Eur. J. Canc.*, **1995**, *31*(A)(5), 576.
[67] Mairs, R.J.; Cunningham, S.H.; Russell, J.; Armour, A.; Owens, J.; McKellar, K; Gaze, M. *J. Nucl. Med.*, **1995**, *36*(6), 1088.
[68] Boyd, M.; Ross, S.; Owens, J.; Hunter, D.; Babich, J.; Zalutsky, M.R.; Hamilton, T.G.; Bell, S.; Mairs, R.J. *Lett. Drug Des. Disc.*, **2004**, *1*(1), 1.
[69] Boyd, M., Spenning, H.S.; Mairs, R.J. *Curr. Gene Ther*., **2003**, *3*(4), 319.
[70] Mairs, R.J.; Boyd, M. *J. Biomed. Biotech.*, **2003**, 2003(2), 102.
[71] Djordjevic, B. *Bio. Essays*, **2000**, *22*, 286.
[72] Belyakov, O.V.; Malcolmson, A.M.; Folkard, M.; Prise, K.M.; Michael, B.D. *Br. J. Cancer*, **2001**, *84*, 674.
[73] Hall, E.J. *Health Phys.*, **2003**, *85*(1), 31.
[74] Wheldon, T.E.; O'Donoghue, J.A.; Barrett, A.; Michalowski, A.S. *Radiother. Oncol.*, **1991**, *21*(2), 91.
[75] Gaze, M.N.; Mairs, R.J.; Boyack, S.M.; Wheldon, T.E.; Barrett, A. *Br. J. Cancer*, **1992**, *66*(6), 1048.
[76] Allen, B.J. *Australas. Radiol.,* **1999**, *43*(4), 480.
[77] Zalutsky, M.R.; Vaidyanathan, G. *Curr. Pharm. Des.*, **2000**, *6*(14), 1433.
[78] Imam, S.K. Int J Radiation Oncology Biol Phys, **2001**, *51*(1), 271.
[79] Jurcic, J.G.; Larson, S.M.; Sgouros, G.; McDevitt, M.R.; Finn, R.D.; Divgi, C.R.; Ballangrud, A.M.; Hamacher, K.A.; Ma, D.; Humm, J.L.; Brechbiel, M.W.; Molinet, R.; Scheinberg, D.A. *Blood*, **2002**, *100*, 1233.

Received: 05 April, 2005 Accepted: 10 August, 2005