Non-invasive in vivo imaging with radiolabelled FIAU for monitoring cancer gene therapy using herpes simplex virus type 1 thymidine kinase and ganciclovir

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Abstract. An experimental cancer gene therapy model was employed to develop a non-invasive imaging procedure using radiolabelled 2'-fluoro-2'-deoxy-5-iodo-1-β-D-arabinofuranosyluracil (FIAU) as an enzyme substrate for monitoring retroviral vector-mediated herpes simplex virus type 1 thymidine kinase gene (*HSV1-tk*) transgene expression. Iodine-131 labelled FIAU was prepared by a no-carrier-added (n.c.a.) synthesis process and lyophilised to give "hot kits". The labelling yield was over 95%, with a radiochemical purity of more than 98%. The stability of [^{131}I]FIAU in the form of lyophilised powder (the hot kit) was much better than that in the normal saline solution. The shelf life of the final [^{131}I]FIAU hot kit product is as long as 4 weeks. Cellular uptake of [^{131}I]FIAU after different periods of storage was investigated in vitro with *HSV1-tk*-retroviral vector transduced NG4TL4-STK and parental non-transduced NG4TL4 murine sarcoma cell lines over an 8-h incubation period. The NG4TL4-STK cells accumulated more radioactivity than NG4TL4 cells in all conditions, and accumulation increased with time up to 8 h. The kinetic profile of the cellular uptake of n.c.a. [^{131}I]FIAU formulated from the lyophilised hot kit or from the stock solution was qualitatively similar. For animal model cancer gene therapy studies, FVB/N mice were inoculated subcutaneously with the *HSV1-tk*(+) and *tk*(−) sarcoma cells into the flank to produce tumours. Biodistribution studies showed that tumour/blood ratios were 2, 3.5, 8.2 and 386.8 at 1, 4, 8 and 24 h post injection, respectively, for the *HSV1-tk*(+) tumours, and 0.5, 0.5, 0.7 and 5.4, respectively, for the *HSV1-tk*(−) tumours. Radiotracer clearance from blood was completed in 24 h and was bi-exponential. A significant difference in radioactivity accumulation was revealed among the *HSV1-tk*(+) tumours, the *tk*(−) tumours and other tissues. At 24 h p.i., higher activity retention was observed in *HSV1-tk*(+) tumours (9.67%±3.89%ID/g) than in *HSV1-tk*(−) tumours (0.48%±0.19%ID/g). After seven consecutive daily treatments with the prodrug ganciclovir, planar gamma camera imaging showed *HSV1-tk*(+) tumour regression at day 4, and complete tumour regression at day 7. These results clearly demonstrate that the simplified n.c.a. synthesis process developed in this study is reliable and that the [^{131}I]FIAU product is useful for in vivo monitoring of *HSV1-tk* gene transfer, expression and gene therapy.

Keywords: [^{131}I]FIAU – Non-invasive imaging – Gene therapy – Herpes simplex virus – Thymidine kinase – Ganciclovir

Introduction

Monitoring gene expression in vivo to evaluate the gene therapy efficacy is a critical issue for scientists and physicians. Non-invasive nuclear imaging can offer information regarding the level of gene expression and its location when an appropriate reporter gene is constructed in the therapeutic cassette [1]. Two approaches in non-invasive imaging development include: (1) enzyme-mediated trapping, i.e. by transducing the cells with reporter genes that encode enzymes able to phosphorylate or metabolise the radiolabelled substrates, so that the metabolites are

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subsequently trapped in transduced cells [2, 3, 4, 5]; (2) receptor-ligand-mediated trapping on the cell surface [6, 7]. Radiolabelled substrates or ligands (reporter probe or marker substrate) are then used for imaging to determine the expression of reporter gene with a gamma camera, single-photon emission tomography (SPET) or positron emission tomography (PET), depending on the isotope used [8, 9, 10]. If the reporter gene is co-localised with a therapeutic gene, the expression of the therapeutic gene could be monitored indirectly.

Herpes simplex virus type 1 thymidine kinase gene (*HSV1-tk*) is the most common reporter gene and is used in cancer gene therapy by activating relatively non-toxic compounds, such as acyclovir or ganciclovir (GCV), to induce cell death [11, 12]. Moreover, various radiolabelled nucleoside analogues are used as specific probes for *HSV1-tk* and can be freely transported across cell membranes. When phosphorylated by the transduced *HSV1-tk* gene, the metabolites of probes subsequently accumulate within the transduced cells. Mammalian cellular thymidine kinase, however, shows relatively low phosphorylation activity and low levels of accumulation of these probes in non-transduced cells. Improved sensitivity and specificity have also been demonstrated with the use of a mutant *HSV1-tk* as a reporter gene transferred by adenovirus vector for the purpose of in vivo non-invasive imaging [3].

2'-Fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodo-uridine (FIAU), an anticancer drug widely used in clinical practice, is an analogue of thymidine [13, 14, 15]. In a series of studies using adenovirus vector for gene transfer, Tjuvajev et al. [1, 2, 16] described the appropriate combination of exogenously introduced *HSV1-tk* as a "marker/reporter gene" and radiolabelled FIAU as a "marker substrate/reporter probe" for monitoring gene therapy and gene expression. Successful specific imaging of *HSV1-tk* expression in animal tumour models were accomplished non-invasively with iodine-131 labelled FIAU and a clinical gamma camera system [16], or with iodine-124 labelled FIAU and PET [17]. Recently, Tjuvajev et al. [18] compared the efficiency of three radiolabelled probes [FIAU, 9-[4-fluoro-3-(hydroxymethyl)butyl]guanine (FHBG) and 9-[3-fluoro-1-hydroxy-2-propoxymethyl]guanine (FHPG)] for in vivo imaging of *HSV1-tk* expression with PET, and demonstrated that FIAU is a substantially more efficient probe than FHBG or FHPG for imaging *HSV1-tk* expression, with greater sensitivity and contrast as well as low levels of abdominal background radioactivity at 2 and 24 h. The initial accumulation and the elimination kinetics of radiolabelled FIAU were reported by Haubner et al. [19] in a tumour-bearing mouse model; the results suggested that sufficient tumour/background ratios for in vivo imaging of *HSV1-tk* expression were reached as early as 1 h post injection. Therefore, a simple and ready-to-use method for preparation of this radiopharmaceutical would be of value and importance.

Tjuvajev et al. [16] prepared radiolabelled FIAU from its unsubstituted precursor FAU [1-(2-deoxy-2-fluoro-β-D-arabinofuranisyl)uracil] by direct radioiodination, which gave 93% pure [131I]FIAU [16]. N.c.a. [124I]FIAU was prepared by reacting FAU with [124I]NaI and the synthesis yield was 95% [17]. In an alternative approach, Vaidyanathan and Zalutsky [20], using tributyltin as precursor, prepared [124I]FIAU from FTAU, and more than 90% of radioactivity in the radiochromatogram eluted as a single peak [20]. In this study, we have developed a simplified synthetic methodology using tributylstannyl derivative as the precursor [21] to synthesise radiolabelled FIAU in lyophilised form (the "hot kit"). The simplicity, high radiochemical yield and high stability of n.c.a. [131I]FIAU preparation has been demonstrated. In order to investigate the biological characteristics of n.c.a. [131I]FIAU and the possibility of monitoring cancer gene therapy using retroviral vector-transduced *HSV1-tk* and GCV, in vitro cellular uptake and in vivo animal studies, including biodistribution and planar gamma camera imaging, were performed in *HSV1-tk*-transduced NG4TL4-STK and parental non-transduced NG4TL4 murine sarcoma cell lines. *Note*: *tk* refers to the thymidine kinase gene and TK refers to the expressed enzyme

Materials and methods

General. 2-Deoxy-2-fluoro-3,5-di-*O*-benzoyl-α-D-arabinofuranose and uracil, along with the enzymes alcohol oxidase, catalase and alkaline phosphatase, were purchased from Sigma-Aldrich Corp.(St. Louis, MO, USA). Hexa-*n*-butylditin and bis(triphenylphosphine) were obtained from Strem Chemicals, Inc.(Newburyport, MA, USA.). Hydrogen bromide (33% solution in acetic acid) and iodine monochloride and other chemicals were purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA).

The NMR spectra were recorded with a Bruker AC-300 Spectrometer at a proton frequency of 300 MHz and chemical shifts were expressed in ppm. Thin-layer chromatography (TLC) was conducted using an imaging scanner (System 200, Bioscan). High-performance liquid chromatography (HPLC) was conducted using Waters Model 600, Waters Model 600E pumps, a Waters Model 486 tunable UV detector and a radioisotope detector (Flow Count Detector FC-003, Capintec, Bioscan). Data were collected and analysed using a computer program (CSW, version 1.7, DataApex Ltd.). The radiochemical yields reported are those obtained at the end of synthesis.

Preparation of 2-deoxy-2-fluoro-3,5-di-O-benzoyl-α-d-arabinofuranosyl bromide (2a). Using a modification of a reported procedure [22], we dissolved 2 g (4.3 mmol) of 2-deoxy-2-fluoro-1,3,5-tri-*O*-benzoyl-α-D-arabinfuranose (**1a**) (Fig. 1) in 50 ml dichloromethane. Under a nitrogen atmosphere, hydrogen bromide (33%) in acetic acid (1.25 ml) was slowly added to **1a**. The mixture was stirred at room temperature overnight. After cooling to ambient temperature, the solvent was removed in vacuo. The crude product was purified by silica gel chromatography (eluent: ethyl acetate/hexane =1/14) to yield 1.65 g of the bromo derivative **2a** (91%). ^{1}H-NMR (CDCl$_3$) δ8.2–7.4 (m, 10H, ArH), 6.6 (d, 1H, C'$_1$-H; J$_{F-H}$=12 Hz), 5.6 (d, 1H, C$_2$-H; J$_{F-H}$=50 Hz), 5.5 (dd, 1H, C$_3$-H; J$_{F-H}$=22 Hz, J=3 Hz), 4.75 (m, 3H, C$_4$-H, C$_5$-H$_2$).

Fig. 1. Synthetic scheme for preparation of organotin precursor FTAU
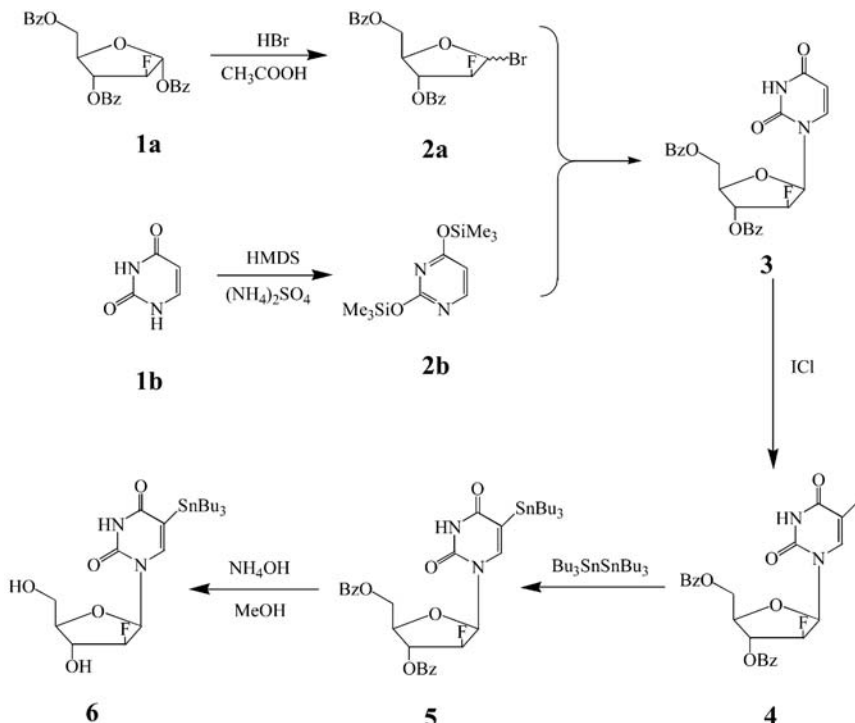
Preparation of 2,4-bis-O-(trimethylsilyl)uracil (2b) [23]. A mixture of uracil (**1b**) (500 mg, 4.46 mmol), ammonium sulphate (0.59 g, 4.46 mmol) and hexamethyldisilazane (10 ml) was refluxed for 4 h. After cooling to room temperature, the clear solution was evaporated under reduced pressure to give a thick oil **2b**, which was used without any further purification.

Preparation of 1-(3,5-di-O-benzoyl-2-deoxy-2-fluoro-β-d-arabino-furanosyl uracil (3). Compound **2a** (1.7 g, 4.3 mmol), dissolved in CH_2Cl_2 (15 ml), was tubing transferred into a flask containing compound **2b** in CH_2Cl_2 (15 ml). The mixture was refluxed overnight. The solvent was removed in vacuo to give a white solid residue. The crude product was purified by silica gel chromatography (eluent: ethyl acetate/hexane =1/1) to give 1.36 g of the white uracil derivative **3** (75%). ^{1}H-NMR ($CDCl_3$) $\delta 8.41$ (br s, 1H, NH), 8.04 (m, 4H, ArH), 7.66–7.41 (m, 7H, ArH; C_6-H), 6.31 (dd, 1H, C_1'-H; J_{F-H}=21.7 Hz, J=2.7 Hz), 5.67 (dd, 1H, C_5-H; J=17.2 Hz, J=2.2 Hz), 5.62 (dd, 1H, C_3'-H; J_{F-H}=18.5 Hz, J=2.7 Hz), 5.31 (dd, 1H, C_2'-H; J_{F-H}=50.4 Hz, J=2.0 Hz), 4.75 (m, 2H, C_5'-H_2), 4.50 (m, 1H, C_4'-H).

Preparation of 1-(3,5-di-O-benzoyl-2-deoxy-2-fluoro-β-d-arabino-furanosyl-5-iodouracil (4). A sample of **3** (260 mg, 0.58 mmol) was dissolved in CH_2Cl_2 (50 ml), and ICl (200 mg) was added. The solution was heated to reflux for 6 h and the solvent was removed under reduced pressure. The crude product was washed with H_2O (50 ml ×2), dried and evaporated. A 265.6 mg (78%) white solid of **4** was obtained after silica gel chromatography purification (eluent: ethyl acetate/hexane =1/1). ^{1}H-NMR ($CDCl_3$) $\delta 8.43$ (br s, 1H, NH), 8.04 (m, 5H, ArH), 7.63–7.43 (m, 6H, ArH; C_6-H), 6.29 (dd, 1H, C_1'-H; J_{F-H}=21.7 Hz, J=2.8 Hz), 5.60 (dd, 1H, C_3'-H; J_{F-H}=18.2 Hz, J=2.6 Hz), 5.35 (dd, 1H, C_2'-H; J_{F-H}=52.8 Hz, J=2.0 Hz), 4.80 (m, 2H, C_5'-H_2), 4.50 (m, 1H, C_4'-H).

Preparation of 1-(3,5-di-O-benzoyl-2-deoxy-2-fluoro-β-d-arabino-furanosyl-5- tributylstannyluracil (5). A mixture of 146.7 mg

(0.25 mmol) of **4**, 261 mg (0.45 mmol) hexabutylditin, and 10 mg bis(triphenylphosphine)palladium dichloride was dissolved in 7.5 ml of anhydrous dioxane. After reflux for 6 h under a nitrogen atmosphere, the dioxane was removed by rotary evaporation. The light green precipitate was filtered through celite. The filtrate was adsorbed onto silica gel and purified by silica gel gradient chromatography (eluent: ethyl acetate/hexane =1/3 to 100% ethyl acetate). The solvent was removed in vacuo to give a white solid of **5** (98.4 mg, 53% yield). ^{1}H-NMR ($CDCl_3$) $\delta 8.89$ (s, 1H, NH), 8.04–7.34 (m, 11H, ArH; C_6-H), 6.33 (dd, 1H, C_1'-H; J_{F-H}= 21.5 Hz, J=2.6 Hz), 5.65 (dd, 1H, C_3'-H; J_{F-H}=18.8 Hz, J=2.8 Hz), 5.33 (dd, 1H, C_2'-H; J_{F-H}=52.8 Hz, J=2.0 Hz), 4.70 (m, 2H, C_5'-H_2), 4.48 (m, 1H, C_4'-H), 1.37–0.78 (m, 27H, $SnBu_3$).

Preparation of 5-tributylstannyl-1-(2-deoxy-2-fluoro-β-d-arabino-furanosyl)uracil (FTAU) (6). The above product **5** (52.7 mg) was added to 10 ml of conc. NH_4OH/MeOH =80/20 solution. The mixture was stirred for 24 h and the solvent was removed under reduced pressure. The crude oil product was purified by silica gel chromatography (eluent: chloroform/hexane =11/1) to afford 33 mg of **6** (87%). ^{1}H-NMR ($CDCl_3$) $\delta 7.48$ (d, 1H, C_6-H; J=2.0 Hz), 6.24 (dd, 1H, C_1'-H; J_{F-H}=18 Hz, J=3.9 Hz), 5.04 (dm, 1H, C_2'-H; J_{F-H}=50 Hz), 4.29 (dm, 1H, C_3'-H; J_{F-H}=16 Hz), 3.92–3.70 (m, 3H, C_4'-H; C_5'-H_2), 1.54–0.85 (m, 27H, $SnBu_3$). The elemental analysis of compound **6** was: FTAU (determined): C, 47.10%; H, 8.53%; N, 5.62%, and FTAU (theoretical): C, 47.13%; H, 6.92%; N, 5.24%.

N.c.a. synthesis of [131I]FIAU from tributyltin precursor FTAU. N.c.a. 5-[^{131}I]iodo-1-(2'-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil ([^{131}I]FIAU) was synthesised from its organotin precursor (Fig. 2). One hundred microlitres of oxidising agent (H_2O_2:1 N HCl:H_2O=4:1:95) was added to a 300-μl V-vial coated with 15 μg of 5-tributylstannyl-(2'-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil (FTAU) and containing 20 μl ethanol and 0.1–5.0 mCi sodium
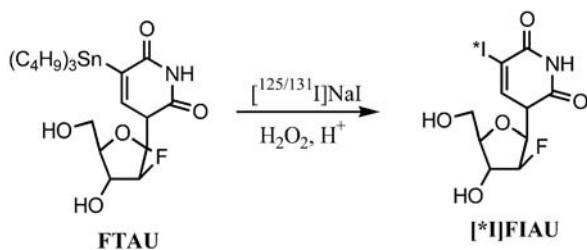
Fig. 2. The preparation of no-carrier-added [131I]FIAU from organotin precursor

[131I]iodide. The reaction mixture was vortexed intermittently. After 8 min, the mixture was frozen in dry ice bath, and then lyophilised with a vacuum system (equipped with a charcoal absorber) to give the final product as a "hot kit". The lyophilised [131I]FIAU hot kit was redissolved in ethanol and the radiochemical purity was determined using TLC and HPLC. TLC was performed on TLC aluminium sheet (Silica gel 60F$_{254}$, Merck), using ethyl acetate/ethanol (90/10, v/v) as the mobile phase. Chromatograms were recorded using an imaging scanner (system 200, Bioscan). HPLC analysis was performed on a reversed-phase column (RPR-1, Hamilton), using methanol/water (50/50, v/v) as the eluent at a flow rate of 1 ml/min. A UV detector (tunable absorbance detector 486, Waters) and a radiodetector (Capintec, Bioscan) were used to analyse the eluate. Data were collected and analysed using computer software (CSW, version 1.7, DataApex Ltd.). The lyophilised [131I]FIAU product, dissolved in physiological saline and eluted through a 0.22-μm apyrogenic disk, was ready for biological or clinical application.

The theoretical specific activity of [131I]FIAU prepared from n.c.a. synthesis process can be calculated from the equation:

$$\text{Specific activity} = Av / (R \times t_{1/2}) \tag{1}$$

where Av is Avogadro's number 6.02×10^{23}, R is the conversion factor 3.7×10^{10} Bq/Ci, $t_{1/2}$ is the physical half-life of the radionuclide in seconds. The theoretical specific activity for [131I]FIAU is $\sim 2 \times 10^7$ Ci/mol.

Stability of n.c.a. [131I]FIAU obtained from the simplified synthesis process. In order to evaluate the stability of n.c.a. [131I]FIAU obtained from the simplified synthesis process, the radiochemical purity of [131I]FIAU in the lyophilised hot kit product and in the normal saline solution were determined at 1, 2, 3, 5, 7, 10, 14, 21 and 28 days after preparation.

Cellular uptake of n.c.a. [131I]FIAU. Two murine cell lines (NG4TL4 and NG4TL4-STK) were used to evaluate n.c.a. [131I]FIAU from precursor FTAU. The NG4TL4-STK cell line, as described previously [24], was derived from the parental NG4TL4 sarcoma cell [25, 26] by transfection with packaged virions of a bicistronic retroviral vector constructed to contain *HSV1-tk* gene, which carries its own promoter, and *neo*R gene, which carries simian virus 40 early promoter [27, 28]. NG4TL4 and NG4TL4-STK cells were cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, 10 μg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere with 5% CO$_2$ at 37°C.

For cellular uptake assay, cells of each cell line were trypsinised and grown overnight in 24-well culture plates (3×10^5 cells/0.5 ml/ well), and the medium was changed before experiment. N.c.a. [131I]FIAU (0.08 μCi/0.5 ml/well) was added to each

well and incubated at 37°C for 15 min, 30 min, 1 h, 2 h, 4 h and 8 h. Triplicates were performed at each time point. For [131I]FIAU uptake assay, the supernatants were removed and the cells rinsed with 200 μl HBSS. Then, cells in each well were harvested with 100 μl of trypsin-EDTA and washed twice with 150 μl HBSS. Cellular uptake of [131I]FIAU was determined by gamma counting in a Wallac 1470 Wizard gamma counter.

Biodistribution of n.c.a. [131I]FIAU. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Taipei Medical University. Syngenic female FVB/N inbred strain mice were inoculated with 1×10^5 NG4TL4 or NG4TL4-STK sarcoma cells subcutaneously to form tumours in the flank. Ten days later, n.c.a. [131I]FIAU (0.01 mCi/animal) was injected via the tail vein. Groups of three animals were sacrificed at 1, 4, 8 and 24 h post injection. Dissected tissue of interest was harvested, washed, weighed and counted along with injection dose standards using a Wallac 1470 Wizard gamma counter.

Planar imaging. Planar imaging was performed on the syngenic FVB/N inbred strain mice bearing subcutaneous NG4TL4-STK tumours in the flank, derived from 1×10^5 NG4TL4-STK cells as described above. All developed palpable tumours were around 10 mm in diameter. After the injection of n.c.a. [131I]FIAU (0.01 mCi/animal) via the tail vein, static images were obtained from anaesthetised animals at day 1, 4, 6 and 7 on a digital gamma camera (Elscint SP-6, Haifa, Israel), equipped with a high-energy pinhole collimator. The image acquisition was performed at 100 kcounts per frame at day 1. The subsequent imaging on days 4, 6 and 7 was acquired using a preset-time acquisition mode. To permit evaluation of tumour regression, animals were treated with GCV (10 mg/kg daily) or 0.09% NaCl by intraperitoneal injection for 7 consecutive days [24, 28, 29].

Results

The preparation and stability of n.c.a. [131I]FIAU

The final product, lyophilised [131I]FIAU hot kit, was redissolved in ethanol and the radiochemical purity was determined using TLC and HPLC. TLC showed the R$_f$ value of [131I]FIAU to be 0.82–0.83 (Fig. 3a). The retention time of [131I]FIAU was 12.91 min by HPLC analysis (Fig. 3b), the same as that obtained from the authentic FIAU standard. The labelling yield was more than 95% and the radiochemical purity was more than 98% (average from more than 10 runs). The stability of n.c.a. [131I]FIAU in the lyophilised hot kit product was shown to be significantly better than that in the normal saline solution by TLC assay. The shelter life of the final [131I]FIAU hot kit product is as long as 3 weeks (Table 1).

Cellular uptake of n.c.a. [131I]FIAU

To evaluate the stability of n.c.a. [131I]FIAU, a cellular uptake study was performed in *HSV1-tk*-transduced NG4TL4-STK and non-transduced parental NG4TL4

Fig. 3a, b. TLC and HPLC analysis demonstrated high radiochemical purity and specific activity. **a** TLC was performed on TLC aluminium sheet, using ethyl acetate/ethanol (90/10, v/v) as the mobile phase. The R_f value of [131I]FIAU was 0.82–0.83. **b** Quality control of the FAU labelling with radio-HPLC, using methanol/water (50/50, v/v) as the eluent at a flow rate of 1 ml/min. The retention time of [131I]FIAU was 12.91 min, the same as that obtained from the cold FIAU standard. The labelling yield was more than 95% and the radiochemical purity was more than 98%
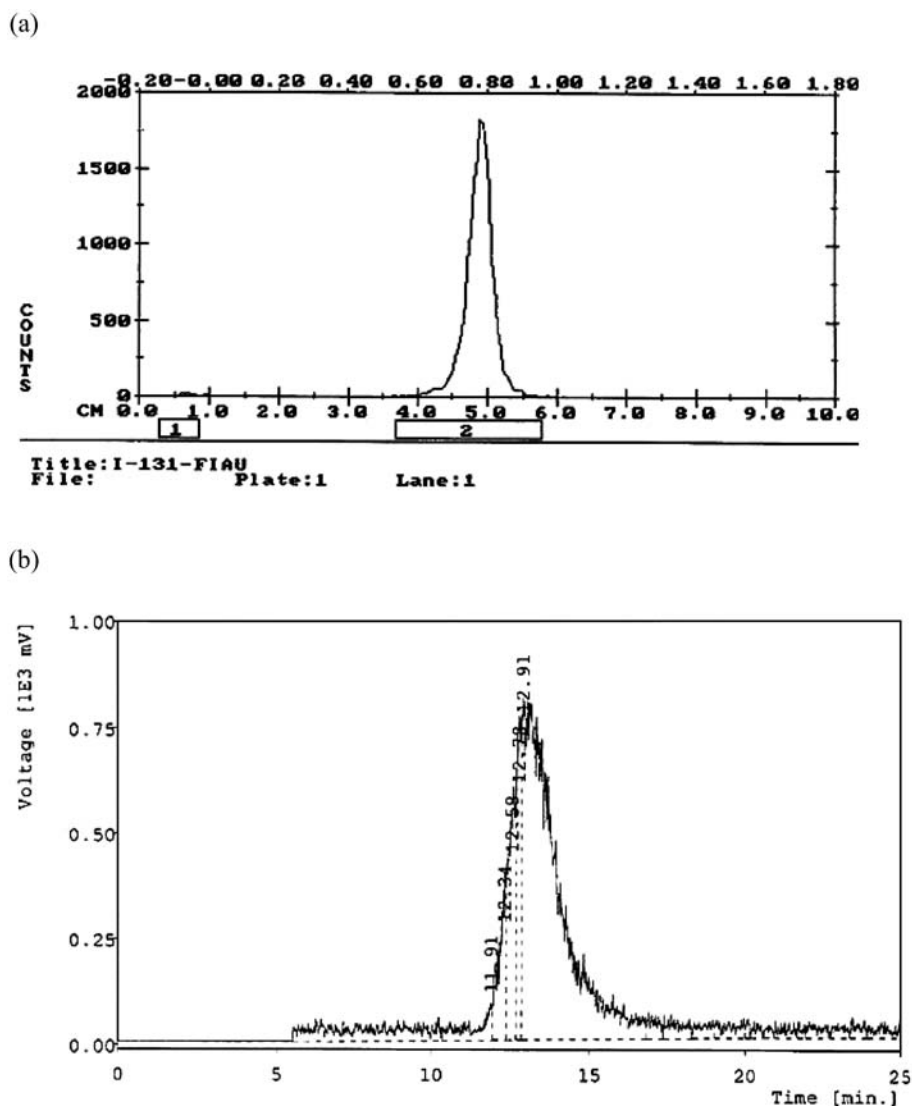

(a)

(b)

Table 1. Stability of n.c.a. [131I]FIAU according to duration of storage[a]

Form of [131I]FIAU	Duration of storage (days)									
	0	1	3	5	7	10	14	17	21	28
In lyophilised hot kit	>99.5	>99.5	>99.5	>99.5	>99.5	>99.5	>99.5	99.1	98.5	97.7
In normal saline solution	>99.5	97.8	95.4	91.3	89.3	84.1	–	–	–	–

No stabiliser was added to either the lyophilised [131I]FIAU hot kit or the stock [131I]FIAU solution
The radioactivity of [131I]FIAU in the lyophilised hot kit or aqueous solution was 100–200 µCi/ml
[a] Values are radiochemical purity in %

murine sarcoma cell lines, which have been employed for investigation of suicidal prodrug cancer gene therapy in previous studies [24, 27, 28]. Figure 4 shows in vitro cellular uptake of n.c.a. [131I]FIAU in NG4TL4-STK and NG4TL4 cells over an incubation period of 8 h. The [131I]FIAU was formulated from the lyophilised hot kit in cold storage for 1 day (a), 7 days (b) or 28 days (c), or

from the [131I]FIAU stock solution that was kept in the refrigerator for 28 days (d).

Compared with the parental NG4TL4 cells that contain no *HSV1-tk* gene, the *HSV1-tk*-transduced NG4TL4-STK cells accumulated more radioactivity under all experimental conditions, and the [131I]FIAU accumulation increased with time up to 8 h after exposure. The kinetic

Fig. 4a–d. Evaluation of the stability of n.c.a. [^{131}I]FIAU. In vitro cellular uptake, in murine sarcoma cell lines NG4TL4-STK and NG4TL4, of [^{131}I]FIAU formulated from the lyophilised hot kit kept in cold storage for 1 day (**a**), 7 days (**b**) or 28 days (**c**), or from the [^{131}I]FIAU stock (normal saline) solution that was kept in the refrigerator for 28 days. The cellular uptake is shown over a period of 8 h
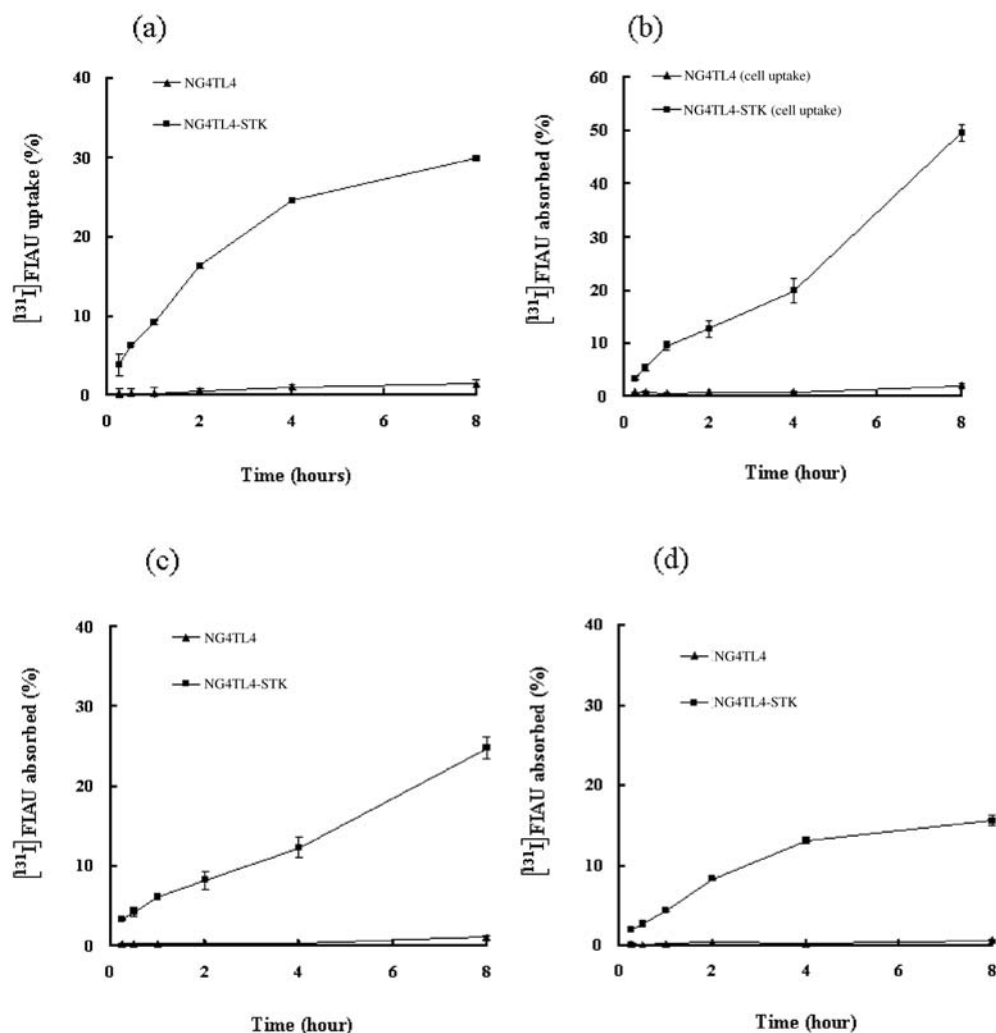
profiles of the cellular uptake of n.c.a. [^{131}I]FIAU formulated from the lyophilised hot kit and from the stock solution were qualitatively similar.

Biodistribution and tumour/blood ratio

The accumulation of n.c.a. [^{131}I]FIAU in tumours and normal tissues was determined by ex vivo gamma counting. FVB/N mice were inoculated subcutaneously with *HSV1-tk*(+) NG4TL4-STK and *tk*(−) NG4TL4 cells into the flank and 10 days later were injected with [^{131}I]FIAU through the tail vein. Biodistribution studies were performed at 1, 4, 8 and 24 h post injection (Fig. 5). As shown in Fig. 5b, the NG4TL4-STK tumour tissues retained consistently higher radioactivity than all other organs at every time point. The *HSV1-tk*(+) tumour/blood ratio reached a maximum at 24 h p.i. At this time point, the retained ^{131}I radioactivity was 9.67%ID/g in NG4TL4-STK tumours and 0.48%ID/g in non-transduced NG4TL4 tumours. Tracer clearance from blood was completed in 24 h. Also, the highest activity of trac-

er accumulation in *HSV1-tk*(+) tumours was observed at 24 h p.i.

The tumour/blood ratios at 1, 4, 8 and 24 h were 2.0, 3.5, 8.2 and 386.8, respectively, for the *HSV1-tk*(+) tumours, and 0.5, 0.5, 0.7 and 5.4, respectively, for the *HSV1-tk*(−) control tumour (Table 2). The kinetics of tissue clearance is shown in Fig. 6. Compared with the blood, liver and tumour(tk−) tissue, NG4TL4-STK tumour(tk+) showed high and lasting accumulation during the study period (Fig. 6a). The retention of radioactivity in the blood, liver and NG4TL4 tumour(tk−) showed only biphasic elimination characteristics (Fig. 6b).

Tumour regression and planar gamma imaging studies

The low non-target tissue uptake of radioactivity and high tumour(tk+)/blood ratios suggested that the NG4TL4-STK tumours can be imaged scintigraphically with n.c.a. [^{131}I]FIAU. Pinhole imaging after intravenous injection of n.c.a. [^{131}I]FIAU clearly reflected the relatively high level of radioactivity retained in transduced

Fig. 5a, b. Biodistribution of [131I]FIAU in mice bearing NG4TL4-STK and NG4TL4 tumours. Radioactivity, as % dose per gram tissue, in **a** NG4TL4 and **b** NG4TL4-STK tumour-bearing mice 1 h, 4 h, 8 h and 24 h following injection of [131I]FIAU
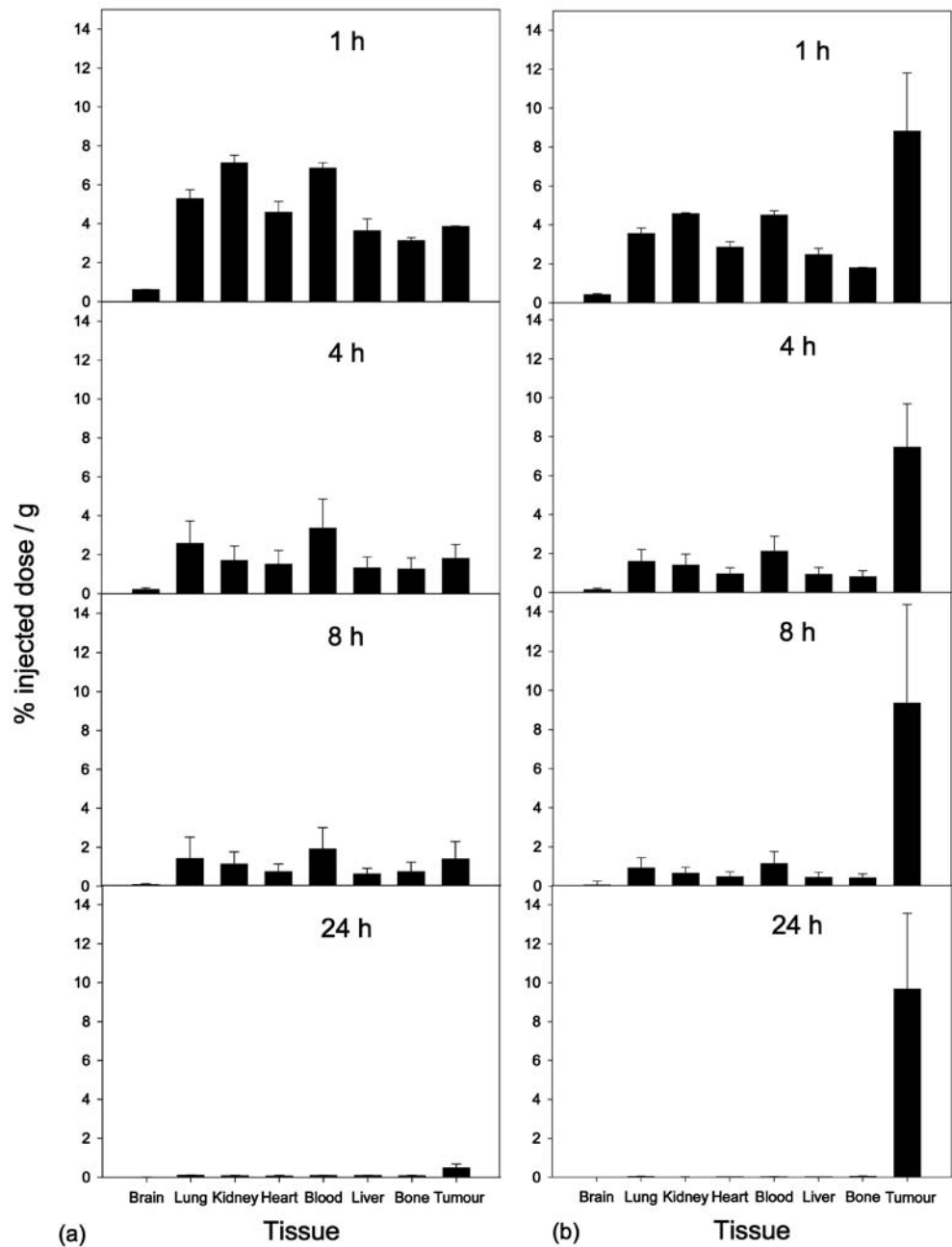

**Table 2.** The kinetic tumour/blood ratio of the NG4TL4-STK (+) and NG4TL4 (−) tumours

Time post injection (h)	Tumour(+)/blood	Tumour(−)/blood
1	2.0±0.77	0.5
4	3.5±1.7	0.5
8	8.2±5.8	0.7
24	386.8±221.7	5.4

tumour(tk+) (Fig. 7). Non-specific uptake in the stomach and thyroid was also demonstrated. After daily GCV treatment, images of the same *HSV1-tk*(+) tumour-bearing mice showed obvious tumour regression at day 4 and virtual tumour disappearance by day 7. As previously noted [28], these mice showed no tumour recurrence nor positive detection by FIAU imaging at the site at up to 60 days of observation (data not shown). The results clearly demonstrated that n.c.a. [131I]FIAU is a promising marker substrate for in vivo monitoring of tumour expression of *HSV1-tk* and of therapeutic effects.

(a).
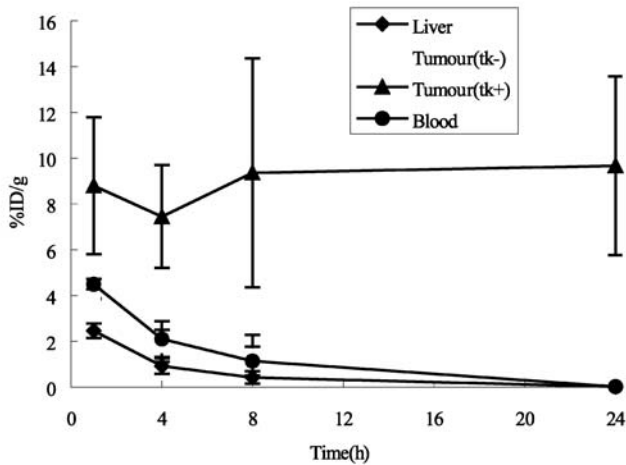
(b)
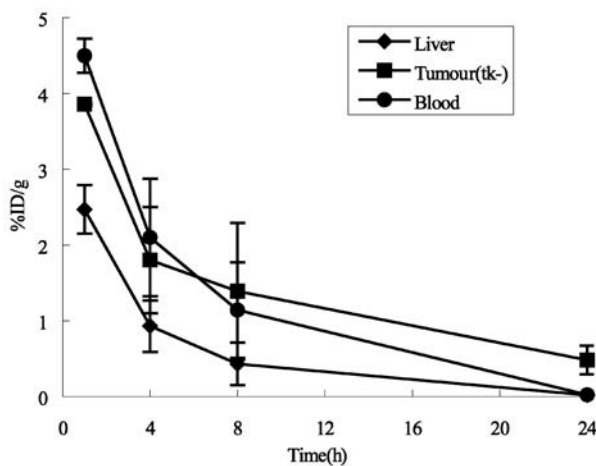
Discussion

More and more gene therapy protocols and clinical trials are in progress, especially in cancer of various types. Thus, it is becoming increasingly important to develop efficient non-invasive methods to monitor the progress of gene transfer and gene expression in vivo. Although elegant exploitation of mutagenised *HSV1-tk* gene showed improved substrate specificity and sensitivity for imaging purposes [3], the use of wild type *HSV1-tk* as a reporter gene with different nucleoside analogues as marker substrates has been investigated. Tjuvajev et al. [18] used [124I]FIAU, [18F]FHBG or [18F]FHPG and PET to compare the efficiency of these three radiolabelled probes for in vivo imaging of *HSV1-tk* expression in transduced RG2 rat gliomas. They reported a 20-fold greater sensitivity of FIAU compared with that of FHBG and a >50-fold sensitivity advantage for FIAU compared with FHPG for imaging *HSV1-tk* expression. In the present study, we employed [131I]FIAU and demonstrated the applicability of this non-invasive imaging method for monitoring of cancer gene therapy in an experimental animal model of *HSV1-tk*-expressing tumour cells transduced with retroviral vector. We followed and modified the procedure of Dougan et. al. [21] to develop a simpler and faster synthetic method for [131I]FIAU. Bearing in mind that the tributylstannyl precursor would be a better choice than the trimethylstannyl precursor owing to the lower toxicity of the tributyltin group compared with trimethyltin [30, 31], we synthesised the tributylstannyl

◀ **Fig. 6a, b.** The kinetics of tissue clearance. **a** Compared with blood, liver and tumour(tk−) tissue, NG4TL4-STK tumour(tk+) showed high and lasting accumulation during the period. **b** In blood, liver and NG4TL4 tumour(tk−), retained radioactivity showed only biphasic elimination characteristics

Fig. 7. Pinhole images of syngeneic FVB/N inbred strain mice bearing subcutaneous NG4TL4-STK (right-sided) tumours. Selective uptake of [131I]FIAU is observed in NG4TL4-STK tumour expressing HSV1-TK (day 1), and there is a progressive reduction in tumour uptake of [131I]FIAU after GCV treatment (days 4, 6 and 7)
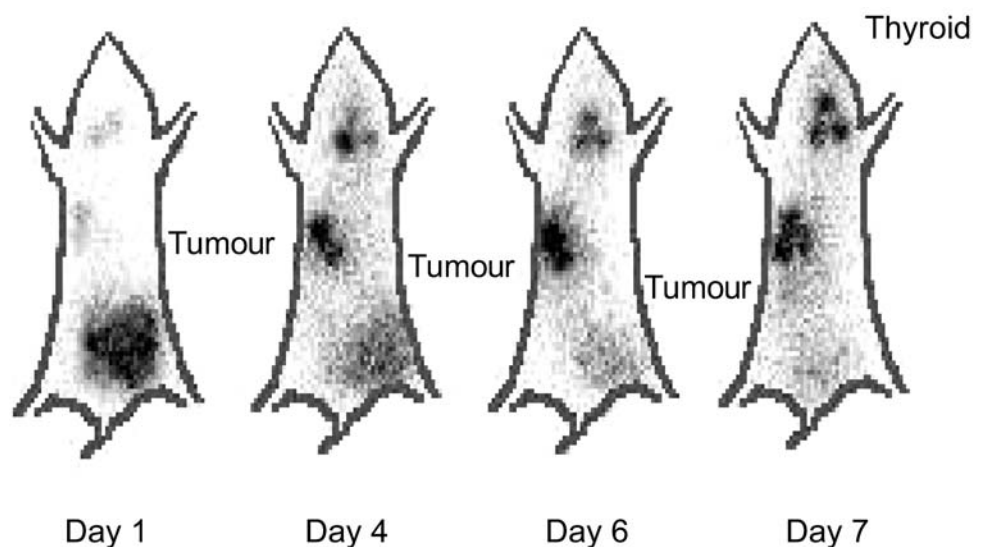
precursor and prepared n.c.a. [131I]FIAU in the form of a lyophilised "hot kit". The convenience and radiochemical yield in [131I]FIAU preparation were significantly improved, and the stability of [131I]FIAU in the lyophilised hot kit product was significantly better than that in normal saline solution.

Although similarly modified procedures have been reported previously [20, 34], the present study showed several advantages of our procedure for preparing radioiodinated tracers from the tributylstannyl precursor:

1. Unreacted [131I]iodide (in the form of [131I]I_2 in the presence of oxidising agent), HCl, solvents (ethanol and H_2O) and oxidising agent (H_2O_2) were all removed during lyophilisation; only [131I]FIAU and a tiny amount of FAU (less than 10 µg) were left in the lyophilised hot kit final product. Hence, no further purification, e.g. using a C-18 Sep-Pak column chromatograph with subsequent methanol elution and removal [34], was needed.
2. The stability of the lyophilised [131I]FIAU hot kit is obvious, and the shelf life is as long as 3 weeks.
3. N.c.a. [131I]FIAU solution with a high radioactivity concentration which is ready for biological application can be readily prepared by dissolving the [131I]FIAU in the hot kit with a small amount of physiological saline.
4. The process has wide application in preparing radioiodinated tracers from their organotin precursors to give a clean, n.c.a. and high radiochemical purity product in high yield. Our [131I]FIAU preparations generally had a radiochemical yield of above 95% and a radiochemical purity of more than 98%, values higher than those reported by Vaidyanathan and Zalutsky (90%) [20] and Tjuvajev et al. (93%) [16]. The higher radiochemical purity may have contributed significantly to successful imaging at earlier time points after tracer injection in this study and that of Haubner et al. [19].

Biodistribution studies were performed at different times post injection in order to examine the kinetics of [131I]FIAU accumulation in vivo. As shown in Fig. 5b, tracer retention and accumulation in the NG4TL4-STK tumour tissues increased with time and reached a maximum at 24 h p.i., while the levels in all other organs remained relatively low. High radioactivity accumulation was observed in the kidneys and the blood in the early phase (1, 4 h) owing to renal excretion. Tracer clearance from blood was completed in 24 h, and extremely low radioactivity accumulation was observed in the brain during this period, presumably due to the blood-brain barrier.

The tissue/blood ratios at 1, 4, 8 and 24 h illustrated the kinetic accumulation level in HSV1-tk(+) tumours. Compared with the HSV1-tk-positive tumours (Table 2), all other organs and the HSV1-tk(−) tumours showed low tracer accumulation at every time point post injection.

The increased accumulation was time dependent and reached a maximum at 24 h after injection. Haubner et al. [19] used [125I]FIAU for in vivo imaging of HSV1-tk gene expression to study the early kinetics of radiolabelled FIAU and reported a bi-exponential tracer clearance from blood. We similarly observed biphasic [131I]FIAU elimination from the blood and organs of sarcoma-bearing mice, except in those with the HSV1-tk(+) NG4TL4-STK tumours. The results showed that the use of [131I]FIAU has little influence on further imaging experiments.

The low non-target tissue uptake of radioactivity and the high tumour/blood ratios suggested that the HSV1-tk-transduced tumours could be imaged scintigraphically with [131I]FIAU. Planar gamma camera imaging after intravenous injection of [131I]FIAU clearly revealed these HSV1-tk gene transduced tumours and the expression level of HSV1-tk was adequate for GCV therapy. Our mouse imaging analysis also showed non-specific uptake in the stomach and thyroid. This observation indicates that the tracer is stable and also resistant to metabolic degradation, which reduces concerns related to imaging of metabolites in both target and non-target tissue.

We also observed contrast between HSV1-tk-expressing tumours tissue and control tumours after GCV treatment. The gamma camera image results clearly demonstrate that image monitoring of cancer gene therapy is possible early after tracer injection. The therapeutic dose of GCV is adequate to match the levels of HSV1-tk expression in tumour tissue. Early imaging for prediction of the efficacy of HSV1-tk with GCV during therapy proved dependable, thus confirming the results of previous studies [24, 27, 28]. However, it remains to be determined whether or not the present in vivo imaging procedure could be applied to assess partial tumour regression as well as recurrence of HSV1-tk transduced tumours.

Recently, PET imaging has been shown to be a preferable system for monitoring gene expression owing to its high sensitivity and quantitative character [2, 32, 33]. Several ongoing studies are being conducted to compare different radiolabelled compounds (pyrimidine nucleoside and acycloguanosine derivatives) in different original cancer cells using SPET and PET imaging systems. These studies will demonstrate the sensitivity, dynamic range and background levels of radioactivity in choosing a reporter probe and in monitoring gene expression. Furthermore, nuclear imaging would be useful for assessment of the efficacy of various cell-based as well as gene-based therapeutic approaches in immunodeficient mice that bear tumours of HSV1-tk-transduced human cancer cells.

In conclusion, a tributyltin precursor can be prepared by using a simple synthetic strategy. [131I]FIAU can be synthesised from this precursor in excellent radiochemical yield. As expected, FIAU not only was stable but also showed high specific uptake in HSV1-tk gene-expressing tumours and fast clearance from normal tissues

of tumour-bearing mice. The optimal time points for imaging range between 2 and 4 h. The results support the view that FIAU may serve as an efficient and selective agent for monitoring of transduced *HSV1-tk* gene expression in vivo in clinical trials.

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