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Current perspective

Evaluation of imatinib mesylate effects on glioblastoma aggressiveness with SPECT radiotracer ^{99m}Tc-(v)-DMSA

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ABSTRACT

In vitro and in vivo studies have demonstrated inhibition of glioblastoma growth by imatinib mesylate (Gleevec®). Imatinib is an inhibitor of the tyrosine kinase activities of platelet-derived growth factor receptor (PDGF-r), which is involved in glioblastoma agressiveness. In this study, we have investigated the link between ^{99m}Tc-(V)-DMSA, an imaging agent used in Single Photon Emission Computed Tomography, cellular accumulation and the biological effects of imatinib mediated by PDGF-r in a human glioblastoma cell line U87-MG. Cells treated with imatinib showed significant decreases in proliferation, invasion, migration and PDGF-rβ expression. ^{99m}Tc-(V)-DMSA cellular uptake studies showed that the specific action of imatinib on PDGF-r signal pathway, in the human glioblastoma cell line U87-MG, could be followed by radioactive tracer. Furthermore, strong correlations between cellular ^{99m}Tc-(V)-DMSA uptake and the effect of imatinib therapy on U87-MG proliferation ($r = 0.896$), invasion ($r = 0.621$) and migration ($r = 0.822$) were obtained, likewise for ^{99m}Tc-(V)-DMSA uptake and PDGF-r expression ($r = 0.958$). Our results show that the biological effects of imatinib therapy on tumour cells properties are linked to PDGF-r phosphorylation and could be traced with ^{99m}Tc-(V)-DMSA, which also seems to be a potential tracer to evaluate the response to imatinib therapy in glioblastoma.

1. Introduction

Glioblastoma is a highly malignant brain tumour with limited therapeutic options, a high recurrence rate and mortality. Because of its uncontrolled proliferation, high degree of invasion, angiogenesis and lack of apopotosis, this tumour type is considered incurable.[1,2] Innovative therapeutic strategies based on recent findings on the molecular genetics of glioblastoma could have an impact on the clinical management of patients with these brain tumours.[3,4] Among these new antitumour drugs, imatinib mesylate (formerly STI571, Gleevec®, Novartis Pharmaceuticals, Basel, Switzerland), was initially proposed for the treatment of chronic myelogenous leukaemia (CML) cells, via selective inhibition of Abl-tyrosine kinase.[5,6]

Results of recent studies have shown that imatinib is also a highly selective inhibitor of the tyrosine kinase activities of c-kit, the tyrosine kinase receptor for stem cell factor (SCF), and platelet-derived growth factor receptor (PDGF-r).[7,8] In particular, imatinib has been reported to inhibit the growth of brain tumours by blocking the PDGF/PDGF-r pathway with a direct action on PDGF-r activity[9,10] PDGF signal pathway is

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known to be involved in the recruitment of protein kinases allowing the activation of pathways for cellular growth, migration, proliferation and survival.[11–13] In light of these findings, it seems that imatinib could have secondary effects on the regulation of proteins involved in glioblastoma aggressiveness. It would therefore be interesting to be able to evaluate the response of glioblastoma to imatinib therapy in order to improve prognosis of patients with this tumour type.

The use of radioactive markers appears to be an interesting in vivo approach to allow early and reliable assessment of treatment response. Several radiotracers are used in nuclear medicine imaging in Single Photon Emission Computed Tomography (SPECT) and among them the pentavalent technetium-99m complex of dimercaptosuccinic acid (^{99m}Tc-(V)-DMSA) has demonstrated a very high affinity for various tumour types, including brain tumours.[14,15] Inflammatory and fibrotic conditions have also been shown to concentrate ^{99m}Tc-(V)-DMSA, due to the acidic environment induced by the presence of white cells and fibroblasts.[16–18] However, the mechanism of ^{99m}Tc-(V)-DMSA cellular uptake is not yet clearly identified. Denoyer and colleagues have recently reported that ^{99m}Tc-(V)-DMSA enters cancer cell lines specifically via type III Na/Pi co-transporters (PiT1) and could reflect on the state of phosphate ion metabolism.[19] Although ^{99m}Tc-(V)-DMSA was described by Koutsikos to be not exclusively a tumour-dependent analog [18], a number of studies have demonstrated its value as a tumour proliferation and aggressiveness marker.[20–22] A recent study using six different cancer cell lines has shown that ^{99m}Tc-(V)-DMSA accumulation is correlated with focal adhesion kinase (FAK) phosphorylation and cellular proliferation that is associated with a poor prognostic. Thus, we have hypothesised a correlation between ^{99m}Tc-(V)-DMSA accumulation and the cellular effects of imatinib treatment through its action on PDGF-r and levels of phosphorylated FAK. The aim of the present study was to investigate in vitro effects of imatinib on the proliferation, migration and invasion capacities of U87-MG cells, which is a widely used model for human malignant glioblastoma. To determine if ^{99m}Tc-(V)-DMSA cell incorporation could be related to the specific cell action of imatinib, the radiotracer uptake was studied in a cytotoxic model that was dependent or independent of PDGF-r.

2. Materials and methods

2.1. Cell culture

The U87-MG human glioblastoma cell line (grade III) was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cell lines were maintained in EMEM supplemented with 10% foetal bovine serum (FCS), according to the manufacturer's instructions in a 5% CO_2 humidified atmosphere. For all assays, quiescent cells were previously incubated overnight in cell culture medium supplemented with 1% FCS to synchronize their cell cycles.

2.2. PDGF receptor tyrosine kinase inhibitor

The 2-phenylaminopyrimidine derivative imatinib was synthesized and kindly provided by Novartis Pharmaceutical AG (Basel, Switzerland). Stock solutions of this compound were prepared at 10 mM in dimethyl-sulfoxide (DMSO) and stored at −20 °C. Dilutions of this stock solution were prepared immediately before use in cell culture medium and added directly to the cells.

2.3. Western blot analysis

After a cell incubation period of 48 h with 20% of foetal bovine serum (FCS), 10 μM and 20 μM of imatinib, cells were lysed in 0.2 ml cell lysis buffer/75 cm^2 culture flask (Cell Signalling Technologies, Ozyme, St-Quentin en Yvelines, France) at 4 °C for 30 min. Lysates were then cleared by a 10 min centrifugation at 1000 ×*g*, and protein determination was carried out using the MicroBCA protein quantification kit (Uptima, Interchim, Montluçon, France). Forty micrograms of each sample was loaded on 8% polyacrylamide–SDS gel and size fractionated by electrophoresis. The separated proteins were transferred to nitrocellulose membrane and incubated with PDGF-rβ primary antibody in 1x Tris Buffered Saline with 0.5% Tween 20 overnight. Primary antibody for PDGF-rβ (*sc-339*, 1:200 dilution) was obtained from Santa Cruz Biotechnology (Santa Cruz, Tebu-bio, Le-Perray-en-Yvelines, France). Actin was detected in each sample by incubating for 1 h with primary antibody against actin clone AC-40 (Sigma, St-Quentin Fallavier, France, 1:2000 dilution) to allow standardization of PDGF-rβ expression by ensuring that equivalent amounts of total protein from each sample was analysed. Membranes were washed and then incubated for 1 h at room temperature with secondary antibody. Bound antibody was detected using UP99619 UptiLight HRP blot substrate (Uptima, Interchim, Montluçon, France). Films were scanned and analysed for quantification with Scion Image free-software (NIH). Normalization of PDGF-rβ signal to levels of actin made it possible to evaluate semi-quantitatively the level of PDGF-rβ expression in each cell line studied.

2.4. Reverse transcription and PCR amplification of cDNA coding for type III Na/Pi co-transporters

Total RNA (tRNA) was extracted from confluent untreated U87-MG cells with Trizol (Life Science Technologies). cDNA was synthesised by reverse transcription of 1 μg of total RNA using 20 U of AMV Reverse Transcriptase (Roche Molecular Biochemical, France) and oligodT as primers (1.6 μg of oligo-p$(dT)_{15}$ Primer; Roche Molecular Biochemical, France). cDNA was amplified in a final volume of 25 μl using 0.625 U of *Taq* DNA polymerase (Promega, France) in the presence of 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide (dNTP), and 0.4 μM of each primer. The sequence of oligonucleotide primers used for amplification for each Na/Pi co-transporter and β-actin is described in Table 1 and was based on the human cDNA sequence for Na/Pi type I (NPT1), Na/Pi type II (NPT2) and Na/Pi type III (PiT1) and β-actin. PCR products were visualised by ultraviolet illumination after electrophoresis on 1% agarose gel containing ethidium bromide. Semi-quantification was performed from video images by densitometry using National Institutes of Health Image software (free software). The amounts of NPT1, NPT2 and PiT1 total RNA were normalised to β-actin as internal standard.

Table 1 – Sequence of primers used to amplify the various na/pi cotransporters and β-actin

NaPi	Sequence	Size of amplified product
NPT1	5′ ACC GAG GCC GAC TTA CTT CTA TGA 3′ 5′ GCT GGA CCA GGG AGG ATG TGA TGT 3′	237 bp
NPT2	5′ GTC ATC CAG AAG GTC ATC AA 3′ 5′ CCC GAG ATG TTG AAG AAG AA 3′	302 bp
PiT1	5′ TAC AAC TCG ACT CAA GGG CTA CTG 3′ 5′ GGG GAC ACG AAC CAA GAC ATC ACA 3′	215 bp
β-actin	5′TTC AAC TCC ATC ATG AAG AAG TGT GAC GTG 3′ 5′ CTA AGT CAT AGT CCG AGA AGC ATT 3′	312 bp

Primers indicated in bold type are complementary to the (+) strand. NPT1, type I NaPi cotransporter; NPT2 type II NaPi cotransporter; PiT1 type III cotransporter.

2.5. *Cell proliferation assay and viability assay*

Growth kinetics and the effects of imatinib on U87-MG glioblastoma cell proliferation were assessed using UptiBlue® (Uptima, Interchim, Montluçon, France).

Cells were seeded in 96-well plates (Greiner Bio-one S.A.R.L., Poitiers, France). Cells were then incubated with imatinib concentrations range from 0 to 100 μM for an appropriate time (24, 48 or 72 h) and 10% v/v UptiBlue® was then added. UptiBlue® reduction was monitored by fluorescence measurements at an excitation wavelength of 530–560 nm and an emission wavelength of 590 nm.

Cell viability and cytotoxic effect of imatinib were monitored by a trypan blue dye exclusion assay. Cells were seeded in 24-well plates (Falcon, Elvetec, France) and incubated with imatinib at IC_{20}: 10–20 μM, or at toxic dose: 50 μM, for 24, 48 and 72 h. Adherent cells were harvested (1% trypsin–EDTA solution) and stained with trypan blue. Dead cells (i.e. those that retained dye) and viable cells (i.e., those that excluded the dye) were counted using low-power microscopy.

2.6. *Cell invasion and migration assays*

After an incubation period of 48 h, in the presence or absence of imatinib at IC_{20} (10–20 μM) glioblastoma cell invasion was evaluated by an in vitro assay, using invasion inserts with an 8 μm pore size (Becton-Dickinson, Bedford, MA). Briefly, the upper surface of each insert was coated with 100 μl of a 1:20 dilution of Matrigel (Becton-Dickinson) and allowed to air-dry overnight. The invasion assay started by adding 50,000 cells in 300 μl of serum-free EMEM medium on the upper surface of coated inserts and 800 μl of 0.2% Bovine Serum Albumin (BSA)-EMEM medium on wells as chemoatractant to induce invasion. After an incubation time of 24 h at 37 °C and 5% CO_2, non-invading cells were removed from the upper surface of the insert by "scrubbing". The numbers of tumour cells which crossed the insert into the lower well and attached to the lower side of insert were quantified by using CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Invitrogen S.A.R.L., Cergy-Pontoise, France) according to the manufacturer's instructions. The percentage of invading cells was calculated by the formula: (mean number of cells migrating in test/mean number of cells migrating in control) × 100%. Glioblastoma cell migration was evaluated as described above except that cells were seeded on the upper surface of uncoated inserts. After incubation time of 4 h at 37 °C and 5% CO_2, cells were quantified, in a similar way as described above, and the results were expressed as a percentage of the number of migrating cells relative to the modulator-free control.

2.7. *Radioactive tracer experiments*

2.7.1. *Preparation of ^{99m}Tc-(V)-DMSA*

^{99m}Tc-(V)-DMSA was prepared according to the method of Yokoyama[23] Radiochemical purity was determined by ascending thin-layer chromatography (TLC) on Merck silica gel 60 F using a mixture of standard *n*-butanol:acetic acid:H_2O (3:2:3) as mobile phase. The radiochemical purity of ^{99m}Tc-(V)-DMSA was greater than 95% in all experiments.

2.8. *Cell kinetic studies*

The cells were plated in 24-well plates (Falcon, Elvetec, France) and incubated with different cytotoxic mediums: a cell-culture medium with 10% FCS or 20% FCS in presence or absence of 10 μM, 20 μM and 50 μM imatinib for 48 h; and a cell-culture medium with 10% FCS or 20% FCS in presence or absence of doxorubicin (10 μM), cisplatin (5 μM) and melphalan (10 μM), in the same conditions of imatinib, at IC_{20} for 48 h. After treatment, cells were used for radioactive tracer uptake as follows: cells were equilibrated in FCS-free medium before adding radioactive tracers; ^{99m}Tc-(V)-DMSA was added to the medium at a concentration of 0.9–1 nM, for a 60 min incubation period, according previous kinetics studies;[24] the cells were rapidly washed three times with phosphate-buffered saline (PBS) at 4 °C; the cells were then solubilised with 1% sodium dodecyl sulfate (SDS) solution in 10 mM sodium borate; and finally the radioactivity in the cellular lysate was counted with a gamma scintillation counter (Packard Cobra 5002, Perkin Elmer, Courtaboeuf, France).

2.9. *Statistical analysis*

Comparisons between untreated control and treated samples were performed. Statistical significance in all experiments

was determined by an unpaired two-tailed Student's t test. P values <0.05 were considered significant. We performed linear regression and calculated the correlation coefficient (r) by using the Pearson test to relate tracer uptake with inhibition of cell proliferation by imatinib.

3. Results

3.1. *In vitro study of the biological effects of imatinib on U87-MG human glioblastoma cell line*

3.1.1. *Evaluation of the effect on PDGF-r expression by western blot analysis*

To determine if malignant glioblastoma cells express PDGF-rβ on their cell surface, we evaluated the protein expression level by western blotting with primary antibodies specific for PDGF-rβ. β-actin expression levels were used to normalise the amounts of PDGF-rβ in the cell line studied. Western blot analysis showed high PDGF-rβ expression in the human glioblastoma cell line tested (Fig. 1a). The PDGF-rβ/β-actin ratio was 0.54 for U87-MG cells. As PDGF-r is a specific target for imatinib, we tested the possible influence of this tyrosine kinase inhibitor on PDGF-rβ expression under three different conditions: after treatment with 10 μM and 20 μM of imatinib and incubation in 20% FCS EMEM medium, which is known to stimulate cells proliferation via the PDGF-r pathway. The results demonstrated a decrease in PDGF-rβ/β-actin ratio in cells treated with 10 μM of imatinib compared to untreated control cells (0.23 vs 0.54) (Fig. 1b). U87-MG cells treated with 20 μM of imatinib also showed a decrease in PDGF-rβ/β-actin ratio compared to untreated control cells (0.17 vs 0.54) (Fig. 1b). In contrast, treatment with a 20% FCS EMEM medium, increased PDGF-rβ expression, with higher PDGF-rβ/β-actin ratio in U87-MG treated than in untreated cells (0.61 vs 0.54) (Fig. 1b).

3.1.2. *Evaluation of cytotoxic effect*

The dose–response curves of imatinib for U87-MG are shown in Fig. 2a. Cell viability was evaluated at 24, 48 and 72 h after treatment. The imatinib concentration associated with 50% inhibition of cell proliferation (IC_{50}) ranged from 42 μM to 88 μM depending on the incubation period: the IC_{50} was 88 μM for an incubation period of 24 h; 67 μM for an incubation period of 48 h; and 42 μM for an incubation period of 72 h (Fig. 2a). The cytotoxic effect was determined for three concentrations lower than IC_{50}: 10, 20 and 50 μM after incubation-periods of 24, 48 and 72 h, and percentage viability and mortality were evaluated (Table 2). Results presented in Table 2 demonstrate that at 10 and 20 μM imatinib, percentage viability was not different from control. In contrast percentage viability was significantly different from control with a concentration of 50 μM (Table 2). For 50 μM the imatinib cytotoxic effect was significant and high cellular mortality was observed. In light of these results and data in the literature, we decided to study the effect of imatinib on U87-MG cell proliferation at the non-toxic (IC_{20}) concentration range of 10–20 μM imatinib with an incubation period of 48 h.

3.1.3. *Evaluation of the effect on cell proliferation*

Imatinib effect on U87-MG cell proliferation during seven days of treatment was evaluated, and the results were standardised according to the proliferation rate by calculating proliferation ratio (Fig. 2b) and comparing it to untreated control (100%). This study showed significant inhibition of cell proliferation. After cell treatment (72 h) with 10 and 15 μM imatinib, we observed 45% and 50% inhibition of cellular proliferation respectively ($P < 0.001$) (Fig. 2b). After a cell treatment of 48 h, with a concentration of 20 μM imatinib, 50% inhibition of cellular proliferation was observed ($P < 0.001$) (Fig. 2b). Inhibition of proliferation remained stable

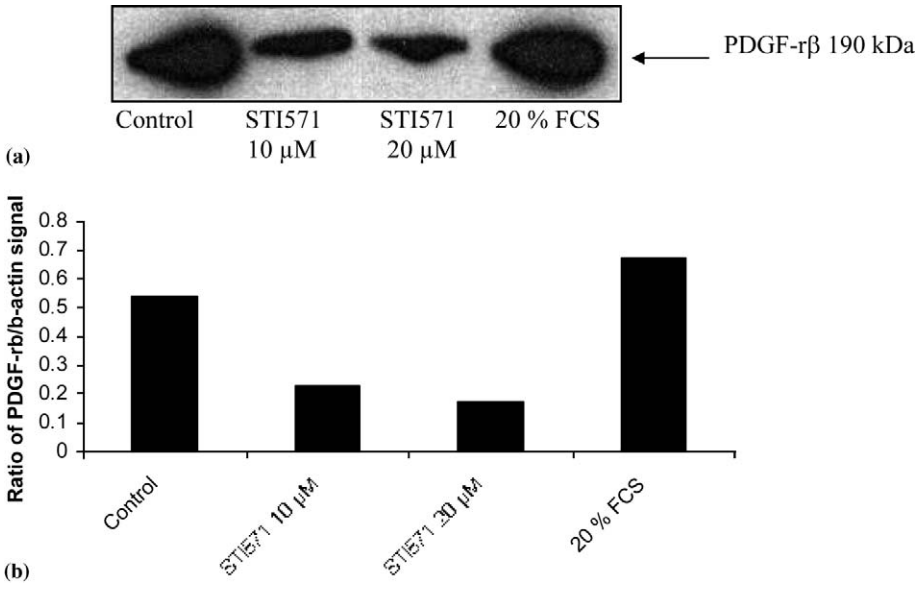

Fig. 1 – (a) Western blot analysis of the imatinib effect on PDGF-rβ expression in U87-MG glioblastoma cells. Equal loading of gels was determined by β-actin expression. The level of PDGF-rβ was determined after loading 40 μg of total protein from cell lysates after treatment with various conditions. (b) Normalization of PDGF-rβ signal to levels of actin, PDGF-rβ signal is presented in relation to β-actin expression level.

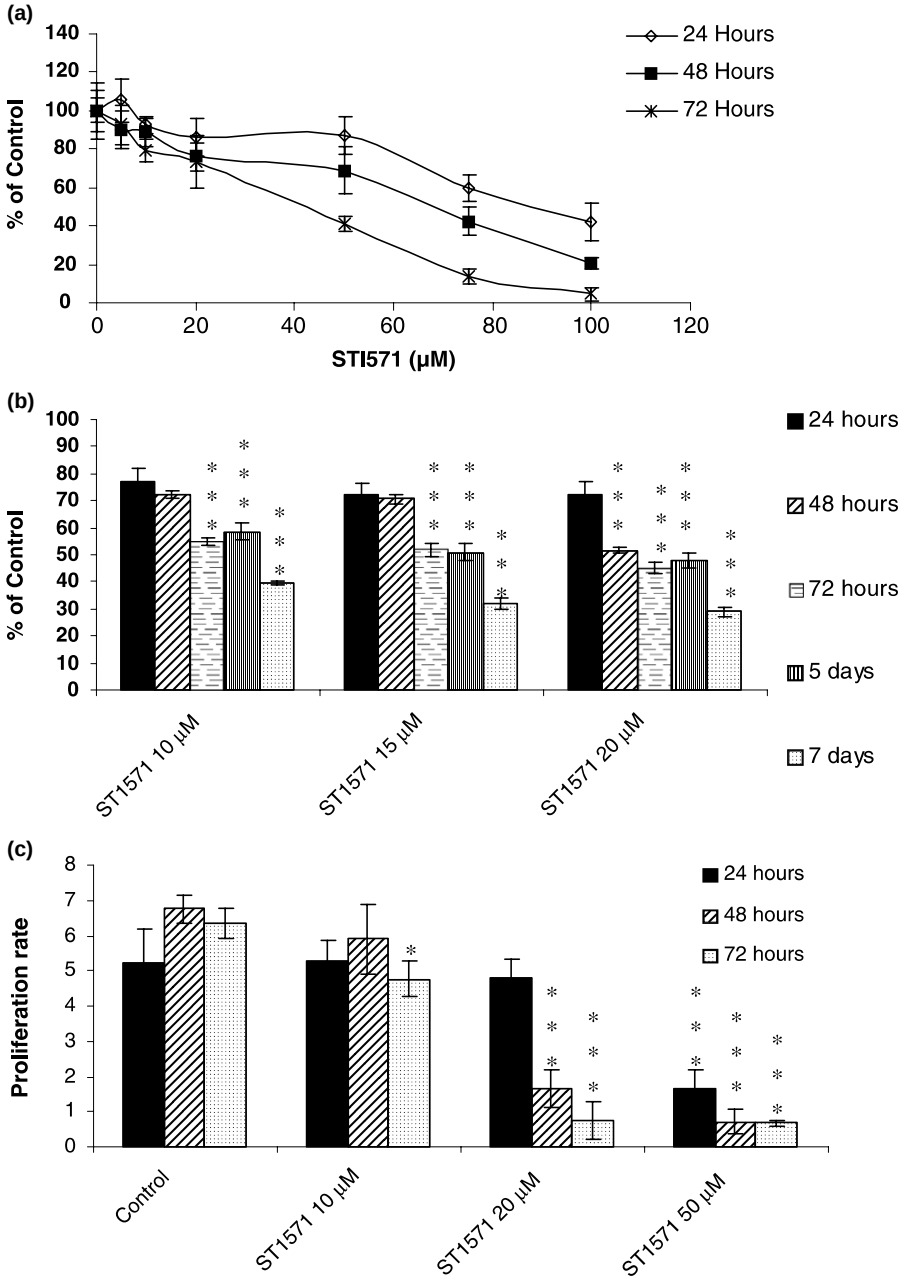

Fig. 2 – Effect of imatinib on viability and proliferation of U87-MG glioblastoma cells. (a) Dose dependent inhibition of u87-mg cell proliferation by imatinib (sti571) cells were incubated for 24, 48 and 72 h with a range of sti571: 0–100 μm. Cell viability and proliferation was accessed by uptiblue® as described in Section 2. each point represents the mean ± standard deviation (SD) of six replicate wells from three independent experiments. (b) Inhibition of proliferation capacity of u-87 mg by imatinib (sti571). Cells were incubated with 10, 15 and 20 μm of sti571. cell proliferation was evaluated with uptiblue® as described in Section 2. each point represents the mean ± SD of six replicate wells from three independent experiments. comparison of untreated control versus each treatment: $^{***}P < 0.001$. (c) Effect of imatinib (sti571) on proliferation rate of u87-mg. cells were incubated for 24, 48 and 72 h with 10, 20 and 50 μm of sti571. Cell proliferation ratio and toxicity was accessed by trypan blue dye exclusion assay as described in Section 2. Each point represents the mean ± SD for six replicate wells.

before declining on the seventh day for the three conditions studied.

Proliferation ratios were evaluated under each concentration for 72 h and results are presented in Fig. 2c. A 72 h treatment with 10 μM of imatinib significantly decreased U87-MG cell proliferation compared to untreated cells (cell proliferation ratio of 4.77 after 10 μM for 72 h vs 6.33 for control, $P < 0.05$; Fig. 2c). At the higher concentration of 20 μM

Table 2 – Evaluation of U87-MG glioblastoma cells viability after appropriate treatment with imatinib (STI571)

	% of cell viability			% of cell mortality		
	24 Hours	48 Hours	72 Hours	24 Hours	48 Hours	72 Hours
Control	88.6 ± 13.23	83.1 ± 11.03	86.8 ± 10.78	11.4 ± 3.83	16.9 ± 2.86	13.2 ± 3.13
STI 10 μM	84.6 ± 13.01	72.5 ± 5.72	77.8 ± 4.82	15.4 ± 2.59	27.5 ± 2.51*	22.2 ± 2.59
STI 20 μM	71.4 ± 6.40	79.0 ± 10.55	81.9 ± 6.67	28.6 ± 3.11*	21.0 ± 4.12	18.1 ± 3.35
STI 50 μM	49.7 ± 6.02**	61.3 ± 9.44*	59.5 ± 6.54**	50.3 ± 9.30***	38.7 ± 7.33**	40.5 ± 4.90***

Cells were incubated for 24, 48 and 72 h with 10, 20 and 50 μM of STI571 and cell viability was accessed by trypan dye exclusion assay as described in Section 2. Each value represents the mean ± SD of mean number of six replicate wells of two independent experiments. Comparison of untreated control versus each treatment: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

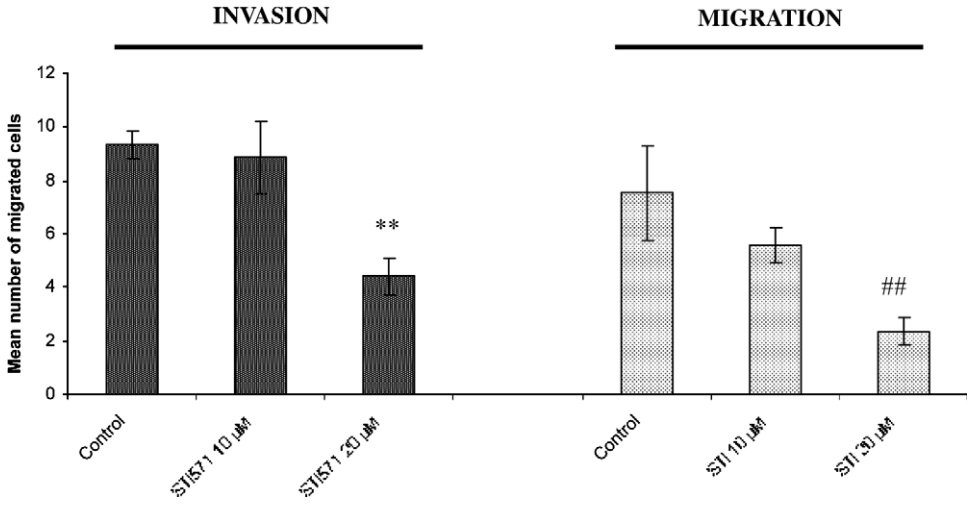

Fig. 3 – Effect of imatinib (STI571) on cell migration and invasion capacity of U87-MG. Cells were incubated with 10 and 20 μM of STI571 for 48 h, and cell invasion and migration assays were performed as described in Section 2. Results were expressed as a mean number of cells which crossed the insert (migrated cells). Each point represents the mean ± SD of mean number of three replicate inserts and is representative of three independent experiments. Comparison of untreated control versus each treatment: *** $P < 0.001$ for invasion capacity and ### $P < 0.001$ for migration capacity.

imatinib for 72 h, cell proliferation ratio decreased as expected. Treatment with 50 μM of imatinib markedly and significantly decreased cell proliferation ratio, and confirmed the toxicity of this dose (Fig. 2c).

3.1.4. *Evaluation of imatinib effects on cell invasion and migration*

Tumour aggressiveness and malignancy is defined by cellular invasion, migration and proliferation capacities. We evaluated the effects of imatinib on U87-MG cell invasion and migration. The effects of imatinib were tested at concentrations of 10 and 20 μM during an exposure time of 48 h, in agreement with the results obtained for the cell proliferation study. As shown in Fig. 3, U87-MG cell invasion and migration were significantly reduced after 48 h of treatment with 20 μM imatinib. The number of invasive cells was decreased by a relative percentage of 53% compared to untreated cells (4.41% of invasive cells with 20 μM vs 9.37% for control, $P < 0.01$; Fig. 3). Similar results were observed for migration capacity with a relative percentage of 69% inhibition at 20 μM imatinib (2.36% of mobile cells with 20 μM vs 7.53% for control, $P < 0.01$; Fig. 3). In contrast, 10 μM imatinib did not have any effect on U87-MG cell invasion and migration compared to untreated cells.

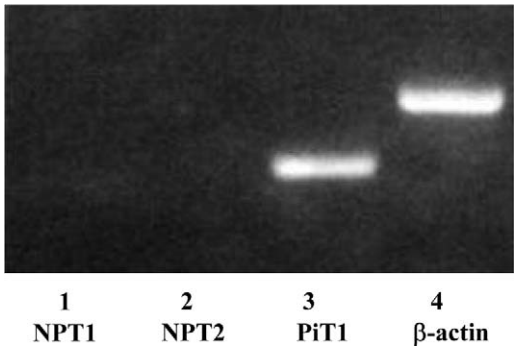

Fig. 4 – RT-PCR characterisation of Na/Pi co-transporter expression in U87-MG glioblastoma cell line. Lane 1: NaPi type I (NPT1) PCR product; Lane 2: NaPi type II (NPT2) PCR product; Lane 3: NaPi type III (PiT1) PCR product; Lane 4: β-actin PCR product.

3.2. *Evaluation of the biological effects of imatinib with ^{99m}Tc-(V)-DMSA*

3.2.1. *Characterization of Na/Pi co-transporter expression by RT-PCR*

We performed PCR using specific primers for each of the three types of Na/Pi co-transporters described and for β-actin to normalise total RNA content of each Na/Pi co-transporter in the cell line studied. PCR performed with cDNA from U87-MG cells did not reveal any PCR product with NPT1 and NPT2 primers (Fig. 4) In contrast, an amplification product of the expected size was obtained with the specific primers for PiT1 (Fig. 4). The PiT1/β-actin ratio was 0.95. The results clearly show that U87-MG cells only expressed type III Na/Pi co-transporters.

3.2.2. *^{99m}Tc-(V)-DMSA uptake*

^{99m}Tc-(V)-DMSA uptake was first studied in presence of various concentrations of imatinib in a classical cell-culture medium, with 10% FCS or in a proliferation-stimulating cell-culture medium with 20% FCS (Fig. 5a). In 10% FCS medium, a 48 h imatinib treatment significantly decreased ^{99m}Tc-(V)-

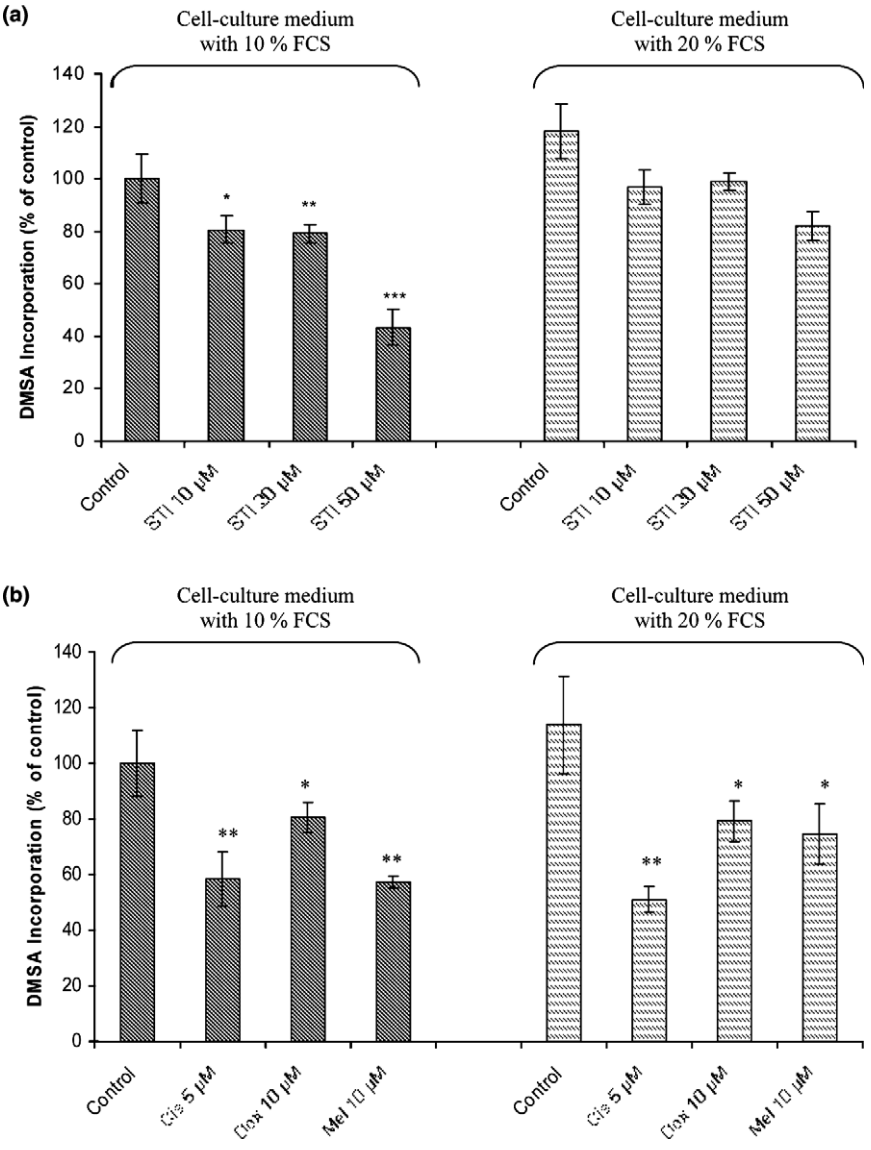

Fig. 5 – Study of ^{99m}Tc-(V)-DMSA incorporation in U87-MG glioblastoma cells. (a) PDGF-r-dependent incorporation of ^{99m}Tc-(V)-DMSA into cells in cytotoxic cell-culture medium. Cells were treated for 48 h with STI571 at 10, 20 and 50 μM in classical (10% FCS) or growth-stimulating (20% FCS) cell-culture medium. Uptake of radioactive tracer ^{99m}Tc-(V)-DMSA was studied. (b) PDGF-r-independent cellular incorporation of ^{99m}Tc-(V)-DMSA in cytotoxic cell-culture medium. Cells were treated for 48 h with various chemotherapeutic drugs at DL20 in classical (10% FCS) or growth-stimulating (20% FCS) cell-culture medium. Cellular uptake of radioactive tracer ^{99m}Tc-(V)-DMSA was studied. Results are expressed as a percentage, where untreated control is 100%. Each point represents the mean ± SD for six replicate wells and is representative of three independent experiments. Comparison of untreated control in a 10% FCS cell-culture medium versus each treatment: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DMSA uptake compared to untreated cells. At concentrations of 20 and 10 μM imatinib, a 20% decrease in ^{99m}Tc-(V)-DMSA uptake was seen, whereas 50 μM imatinib induced a more significant decrease of ^{99m}Tc-(V)-DMSA uptake by about 60% compared to control (Fig. 5a). An increase in cellular ^{99m}Tc-(V)-DMSA uptake was observed after a 48 h incubation in proliferation-stimulating medium (20% FCS). But the combination of proliferation-stimulating medium and imatinib induced a less marked increase in cellular ^{99m}Tc-(V)-DMSA uptake (Fig. 5a). A decrease was even observed with a combination of 20 or 50 μM imatinib and 20% FCS medium, compared to a 20% FCS medium alone (Fig. 5a).

In a different set of experiments, ^{99m}Tc-(V)-DMSA uptake was studied after various chemotherapeutic (cisplatin, doxorubicin and melphalan) treatments in medium supplemented with either 10% or 20% FCS (Fig. 5b). In the 10% FCS medium, a 48 h incubation with all drugs significantly decreased ^{99m}Tc-(V)-DMSA uptake compared to untreated cells. A treatment with 5 μM cisplatin, 10 μM doxorubicin or 10 μM of melphalan decreased the uptake of ^{99m}Tc-(V)-DMSA by 41%, 20% and 43% respectively (Fig. 5b). In 20% FCS medium, a significant decrease in radiotracer uptake of 50%, 21% and 25% after 48 h exposure to cisplatin, doxorubicin and melphalan, could be observed respectively (Fig. 5b). Contrary to the effects observed after an incubation with imatinib, combining 20% FCS medium and different chemotherapeutic drugs induced the same effects on cellular ^{99m}Tc-(V)-DMSA uptake as obtained with 10% FCS medium (Fig. 5b).

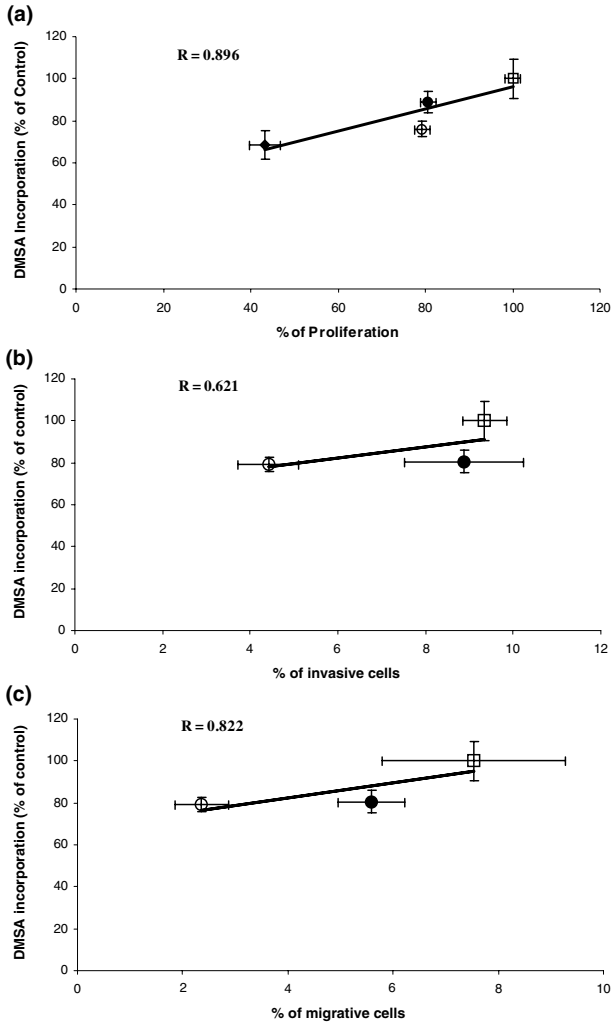

Fig. 6 – Correlation of ^{99m}Tc-(V)-DMSA uptake with (a) U87-MG cell proliferation, (b) cell invasion, and (c) cell migration after incubation with imatinib (STI571). Results are expressed as the percentage of ^{99m}Tc-DMSA (V) incorporation, compared to control considered as 100%, and as the percentage of cell proliferation, invasion and migration after a 48 h treatment with different concentrations of STI571: (♦) 50 μM, (●) 20 μM, (○) 10 μM and (□) control. Solid lines are linear regressions and r is Person's correlation coefficient.

3.3. *Correlation analysis*

We tried to correlate the percentage of cellular ^{99m}Tc-(V)-DMSA uptake with multiple effects of imatinib treatment on malignant glioblastoma tumourigenesis factors. Modulation of cellular proliferation, invasion and migration was obtained by treating cells with imatinib, as previously described, and was compared with cellular ^{99m}Tc-(V)-DMSA uptake. Determination of the Pearson correlation coefficient (Fig. 6) showed that ^{99m}Tc-(V)-DMSA uptake and the effects of imatinib on cell proliferation, invasion and migration were correlated, with Person correlation coefficients of $r = 0.896$, $r = 0.621$ and $r = 0.822$, respectively. We also tried to established a correlation with ^{99m}Tc-(V)-DMSA uptake and the PDGF-rβ level expression. Simple regression analysis identified a positive correlation between percentage of ^{99m}Tc-(V)-DMSA incorporation and PDGF-rβ/β-actin ratio with a Person correlation coefficient of $r = 0.958$, thus demonstrating a close link between the ^{99m}Tc-(V)-DMSA incorporation, PDGF-rβ expression, treatment by imatinib and cellular proliferation (Fig. 7).

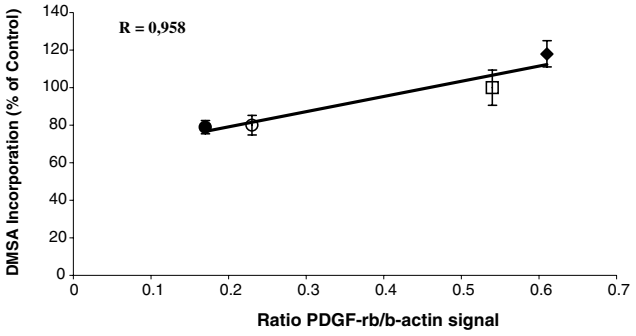

Fig. 7 – Correlation of ^{99m}Tc-(V)-DMSA uptake with the PDGF-rβ expression in U87-MG glioblastoma cells. Results are expressed as the percentage of ^{99m}Tc-DMSA (V) incorporation, compared to control considered as 100%, and as the PDGF-rβ/β-actin ratio after different treatments: (♦) 20% FCS, (●) 20 μM STI571, (○) 10 μM STI571 and (□) control. Solid lines are linear regressions and r is Person's correlation coefficient.

4. Discussion

Imatinib mesylate has been demonstrated to be a potent and selective tyrosine kinase inhibitor.[6–8] More specifically, imatinib has been shown to be an effective inhibitor of cell growth via inhibition of platelet-derived growth factor receptor (PDGF-r) phosphorylation.[8,9], involved in the recruitment of protein kinases allowing cancer progression.[25] In recent studies we have demonstrated that ^{99m}Tc-(V)-DMSA uptake, an imaging agent used in SPECT, is related to phosphate ion transport[19] and could be correlated to the phosphorylated focal adhesion kinase (FAK) level, which is directly linked to the cell proliferation rate.[21,22] Previous studies demonstrated the overexpression of PDGF-r in human glioblastoma and suggested that PDGF-r plays a major role in human gliomagenesis.[26] In the present study, we have explored a possible extension of imatinib applications to human malignant glioblastoma. Experiments were done using the U87-MG cell line, which is described in the literature as an in vitro model of human malignant glioblastoma due to its particularly aggressive characteristics. Western blotting for PDGF-rβ was performed and, in agreement with the literature, demonstrated high expression of PDGF-rβ in U87-MG.[10] Overexpression of PDGF-rβ observed in U87-MG makes this cell type a good target for imatinib, however few studies have evaluated the effect of imatinib on these human malignant glioblastoma cells. An incubation period of two days with 20% FCS medium induced an increase in PDGF-rβ expression in U87-MG cells. In contrast, decreased PDGF-rβ expression level was associated with decreased cell proliferation, which was observed after treatment with imatinib. The protein expression study underlined the specific dose-dependent action of imatinib on PDGF-rβ expression. Our results show a decrease in PDGF-rβ expression after treatment with imatinib, whereas incubation in 20% FCS medium that is rich in growth factors, showed an increase in PDGF-rβ levels, as reported in the literature.[27] The proliferation study demonstrated that imatinib has a dose-dependent and time-dependent inhibitory effect in the cancer cell line tested, with an IC_{50} ranging between 42 and 88 μM according the incubation period. The results of the proliferation study showed a significant inhibition of cell growth, without cell toxicity, after a 48 h treatment with imatinib at a concentration of 20 μM in agreement with those reported in the literature.[28,29] This inhibition of cell proliferation was observed during 7 days. As already described, we conducted the study using the IC_{20} imatinib concentration, which corresponded to a dose that induced significant biological effects without the epi-phenomenon of cellular mortality.[30]

Different studies have underlined the important role of PDGF-r/PDGF autocrine growth loop in glioblastoma tumourigenesis via the involvement of a number of protein kinases cascades, including mechanisms underlying cell proliferation, migration and invasion.[12,13] It is therefore not surprising to observe an inhibition of U87-MG cell invasion and migration following treatment with imatinib, in agreement with the results obtained for other cell types.[31,32] Phosphate ion is known to play an important role in cell proliferation and is also intimately involved in the phosphorylation activity of proteins. ^{99m}Tc-(V)-DMSA has been described as a marker of phosphate transport and enters cancer cell lines specifically via type III Na/Pi co-transporters (PiT1).[19,22] All Na/Pi co-transporter expression in the U87-MG cell line was studied by RT-PCR assay and results demonstrated that U87-MG cells only express PiT1. Study of ^{99m}Tc-(V)-DMSA uptake has shown an inverse correlation between ^{99m}Tc-(V)-DMSA cellular accumulation and drug concentrations in U87-MG cells treated with imatinib in 10% FCS medium. Contrary to this, U87-MG cells in 20% FCS medium showed an increase in ^{99m}Tc-(V)-DMSA uptake. However, after incubation with chemotherapeutic drugs, whose action is independent of PDGF-r expression, we observed a similar decrease in ^{99m}Tc-(V)-DMSA uptake in both 10% and 20% FCS medium. So, our results have clearly proven that the modulation of ^{99m}Tc-(V)-DMSA accumulation is linked to the modulation of PDGF-r signal pathway, due to the specific action of imatinib on PDGF-r. These observations are in agreement with a recent study which showed that the PDGF-r signal could selectively stimulate phosphate transport by regulating the expression of type III Na/Pi co-transporters that also transport ^{99m}Tc-(V)-DMSA [33]. In addition, our study demonstrated a correlation between the various cellular parameters influenced by imatinib treatment, i.e. cell proliferation, migration and invasion and ^{99m}Tc-(V)-DMSA uptake. Strong correlations were observed between cell proliferation ($r = 0.896$), migration ($r = 0.822$) and invasion ($r = 0.621$) after treatment with imatinib and the level of ^{99m}Tc-(V)-DMSA uptake. In the same way, we have established a direct correlation between the PDFG-rβ expression and ^{99m}Tc-(V)-DMSA uptake ($r = 0.958$), demonstrating that imatinib therapy and cell proliferation is linked via PDGF pathway.

In summary, our investigations confirm the therapeutic potential of imatinib treatment in malignant glioblastoma with anti-tumour effects on proliferation, migration and invasion capacities. These results underline the importance of followed-up to treatment efficacy in the management of patients with this tumour type in improving prognosis. A direct correlation between ^{99m}Tc-(V)-DMSA uptake and the cellular effects of imatinib therapy has been established, by the specific action of imatinib on PDGF-r signal pathway, with a possible regulation of PiT1 expression. Thus, ^{99m}Tc-(V)-DMSA could be an interesting non-invasive agent for the evaluation of response to imatinib therapy in malignant glioblastoma, and may be helpful for patients management and treatment monitoring.

Conflict of interest statement

None declared.

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REFERENCES

1. Lemke DM. Epidemiology, diagnosis, and treatment of patients with metastatic cancer and high-grade gliomas of the central nervous system. *J Infus Nurs* 2004;**27**:263–9.
2. Kitange GJ, Templeton KL, Jenkins RB. Recent advances in the molecular genetics of primary gliomas. *Curr Opin Oncol* 2003;**15**:197–203.
3. Boudreau CR, Yang I, Liau LM. Gliomas advances in molecular analysis and characterization. *Surg Neurol* 2005;**64**:286–94.
4. Grossman SA, Batara JF. Current management of glioblastoma multiforme. *Semin Oncol* 2004;**31**:635–44.
5. Druker BJ. Imatinib mesylate in the treatment of chronic myeloid leukaemia. *Expert Opin Pharmacother* 2003;**4**:963–71.
6. Deininger M, Buchunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 2005;**105**:2640–53.
7. Buchdunger E, O'Reilly T, Wood J. Pharmacology of imatinib (STI571. *Eur J Cancer* 2002;**38**:S28–36.
8. Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, et al. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2002;**295**:139–45.
9. Kilic T, Alberta JA, Zdunek PR, Acar M, Iannarelli P, O'Reilly T, et al. Intracranial inhibition of platelet-derived growth factor-mediated glioblastoma cell growth by an orally active kinase inhibitor of the 2-phenylaminopyrimidine class. *Cancer Res* 2000;**60**:5143–50.
10. Hermanson M, Funa K, Hartman M, Claesson-Welsh L, Heldin B, Westermark B, et al. Platelet-derived growth factor and its receptors in human glioma tissue, expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res* 1992;**52**:3213–9.
11. Heldin CH, Westermarck B. Platelet-derived growth factor, mechanism of action and possible in vivo function. *Cell Regul* 1990;**1**:555–66.
12. Kapoor GS, O'Rourke DM. Receptor tyrosine kinase signaling in gliomagenesis, pathobiology and therapeutic approaches. *Cancer Biol Ther* 2003;**2**:330–42.
13. Kapoor GS, O'Rourke DM. Mitogenic signaling cascades in glial tumours. *Neurosurgery* 2003;**52**:1425–34.. discussion 1434–35.
14. Hirano T, Otake H, Kazama K, Wakabayashi K, Zama A, Shibasaki T, et al. Technetium-99m (V)-DMSA and thallium-201 in brain tumour imaging, correlation with histology and malignant grade. *J Nucl Med* 1997;**38**:1741–9.
15. Hirano T, Otake H, Shibasaki T, Tamura M, Endo K. Differentiating histologic malignancy of primary brain tumours, pentavalent technetium-99m-DMSA. *J Nucl Med* 1997;**38**:20–6.
16. Lee BF, Chiu NT, Wu DC, et al. Use of ^{99m}Tc (V)DMSA scintigraphy in the detection and localization of intestinal inflammation: comparison of findings and colonoscopy and biopsy. *Radiology* 2001;**220**:381–5.
17. Athanasoulis T, Koutsikos J, Moulopoulos LA, Tsiouris S, Dimopoulos MA, Zerva C. Reverse of differential uptake intensity of Tc-99m MIBI and Tc-99m V-DMSA by multiple multiple myeloma lesions in response to therapy. *Clin Nucl Med* 2003;**28**:631–5.
18. Koutsikos J, Athanasoulis T, Anagnostopoulos A, Velidaki A, Passadi M, Dimopoulos MA, et al. Combined use of 99m Tc-Sestamibi and 99mTc-V-DMSA in the assessment of chemotherapy effectiveness in patients with multiple myeloma. *J Nucl Med* 2005;**46**:978–82.
19. Denoyer D, Perek N, Le Jeune N, Frere D, Dubois F. Evidence that 99mTc-(V)-DMSA uptake is mediated by Na/Pi co-transporter type III in tumour cell lines. *Eur J Nucl Med Mol Imaging* 2005;**32**:820–7.
20. Papantoniou VJ, Tsiouris S. In vitro verification of the correlation of in vivo 99mTc-(V)DMSA uptake with cellular proliferation rate. *Eur J Nucl Med Mol Imaging* 2005;**32**:1240–1.
21. Denoyer D, Perek N, Le Jeune N, Cornillon J, Dubois F. Correlation between (99m)Tc- (V)-DMSA uptake and constitutive level of phosphorylated focal adhesion kinase in an in vitro model of cancer cell lines. *Eur J Nucl Med Mol Imaging* 2005;**32**:820–7.
22. Papantoniou VJ, Souvatzoglou MA, Valotassiou VJ, Louvrou C, Ambela C, Koutsikos J, et al. Relationship of cell proliferation (Ki-67) to 99mTc-(V)DMSA uptake in breast cancer. *Breast Cancer Res* 2004;**6**:R56–62.
23. Yokoyama A, Hata N, Horiuchi K, Nasuda H, Saji H, Ohta H, et al. The design of a pentavalent 99mTc-dimercaptosuccinate complex as a tumour imaging agent. *Int J Nucl Med Biol* 1985;**12**:273–9.
24. Denoyer D, Perek N, Le Jeune N, Frere D, Dubois F. The multidrug resistance of in vitro tumour cell lines derived from human breast carcinoma MCF-7 does not influence pentavalent technetium-99m-dimercaptosuccinic Acid uptake. *Cancer Biother Radiopharm* 2003;**18**:791–801.
25. George D. Platelet-derived growth factor receptors, a therapeutic target in solid tumours. *Semin Oncol* 2001;**28**:27–33.
26. Plate KH, Breier G, Farrell CL, Risau W. Platelet-derived growth factor receptor-beta is induced during tumour development and upregulated during tumour progression in endothelial cells in human gliomas. *Lab Invest* 1992;**67**:529–34.
27. Beppu K, Jaboine J, Merchant MS, Mackall CL, Thiele CJ. Effect of imatinib mesylate on neuroblastoma tumourigenesis and vascular endothelial growth factor expression. *J Natl Cancer Inst* 2004;**96**:46–55.
28. Te Kronnie G, Timeus F, Rinaldi A, Crescenzio N, Spinelli M, Rosolen A, et al. Imatinib mesylate (STI571) interference with growth of neroectodermal tumour cell lines does not critically involve c-Kit inhibition. *Int J Mol Med* 2004;**14**:373–82.
29. Roussidis AE, Mitropoulou TN, Theocharis AD, Kiamouris C, Papadopoulos S, Kletsas D, et al. STI571 as a potent inhibitor of growth and invasiveness of human epithelial breast cancer cells. *Anticancer Res* 2004;**24**:1445–7.
30. Schiffer D, Cavicchioli D, Giordana MT, Palmucci L, Piazza A. Analysis of some factors effecting survival in malignant gliomas. *Tumouri* 1979;**65**:119–25.
31. Bellone G, Ferrero D, Carbone A, De Quadros MR, Gramigni C, Prati A, et al. Inhibition of cell survival and invasive potential of colorectal carcinoma cells by the tyrosine kinase inhibitor STI571. *Cancer Biol Ther* 2004;**3**:385–92.
32. Kakita A, Suzuki A, Nishiwaki K, Omo Y, Kotake M, Ariyoshi Y, et al. Stimulation of Na-dependent phosphate transport by platelet-derived growth factor in rat aortic smooth muscle cells. *Atherosclerosis* 2004;**174**:17–24.