

Establishment of a Mouse Gastrointestinal Stromal Tumour Model and Evaluation of Response to Imatinib by Small Animal Positron Emission Tomography

HANS PRENEN¹, CHRISTOPHE DEROOSE², PETER VERMAELEN², RAF SCIOT³,
MARIA DEBIEC-RYCHTER⁴, SIGRID STROOBANTS², LUC MORTELMANS²,
PATRICK SCHÖFFSKI¹ and ALLAN VAN OOSTEROM¹

¹Laboratory for Experimental Oncology (LEO), Department of General Medical Oncology,
Departments of ²Nuclear Medicine and ³Pathology and

⁴Center for Human Genetics, Catholic University Leuven, B-3000 Leuven, Belgium

Abstract. *Background: Gastrointestinal stromal tumours (GIST) predominantly express activating mutations of the KIT tyrosine kinase receptor and are successfully treated with imatinib mesylate, a KIT inhibitor. As resistance to imatinib causes therapy failure, our aim was to develop an in vivo GIST model to evaluate KIT inhibitors and monitor therapy with small animal positron emission tomography (PET). Materials and Methods: The first mouse model of GIST xenografts was successfully established by injecting GIST882 cells subcutaneously into nude mice. Results: Using the small animal PET, FDG up-take in xenografts was significantly decreased after 24 h of treatment with imatinib, which correlated with a response to treatment, e.g., with a decrease in tumour volume, the inhibition of KIT and downstream intermediate phosphorylation and arrest of tumour cell proliferation as evaluated after 7 days of treatment. Conclusion: This model is useful to study imatinib resistance and to evaluate novel targeted therapies.*

Gastrointestinal stromal tumours (GIST) are the most common gastrointestinal mesenchymal tumours. They can arise anywhere along the gastrointestinal tract or beyond it in the mesentery or omentum, but are most often located in the stomach and small intestine (1). GISTs are thought to arise from the (precursors of) interstitial cells of Cajal (ICC), the

gastrointestinal pacemaker cells, and are typically characterised by the expression of the type III receptor tyrosine kinase KIT (CD 117) (2). KIT is a 145-kDa transmembrane glycoprotein that is encoded by the proto-oncogene *KIT* and its ligand is stem cell factor (SCF) (3, 4). *KIT* activating mutations are detected in more than 80% of sporadic GISTs. Specifically, up to 70% of GISTs carry mutations at exon 11 of the gene, which encodes the juxtamembrane domain of KIT (5-7). These mutations result in gain of function with activation of the KIT receptor without binding of the stem cell factor ligand and are believed to be the underlying pathogenic event in GIST tumorigenesis (8).

The anti-neoplastic drug imatinib mesylate, a derivative of 2-phenylaminopyrimidine also known as STI571 or Glivec[®], inhibits tyrosine kinases, such as BCR-ABL, PDGFRs and KIT. Multicenter clinical trials have proven that imatinib is highly effective for the treatment of inoperable and/or metastatic GISTs (9-11) and remains, so far, the only effective systemic treatment. In GISTs, imatinib functions through binding to the ATP-binding site in the cytoplasmatic tyrosine kinase domain of KIT, which leads to the inhibition of tyrosine phosphorylation of proteins involved in KIT signal transduction.

Despite the promising clinical results achieved with imatinib treatment in GIST, there is an increasing problem of resistance occurring after a period of imatinib treatment. Therefore, the interest in other signal transduction inhibitors is growing to overcome this resistance. The aims of our study were to establish a GIST mouse model that can be used for the testing of signal transduction inhibitors similar to imatinib and to determine whether response can be evaluated by small animal positron emission tomography (PET). Furthermore, the effect of imatinib was studied in GIST tumours growing in nude mice to investigate their dependency on the oncogenic KIT signalling pathways.

Correspondence to: Hans Prenen, MD, Laboratory for Experimental Oncology, Department of General Medical Oncology, University Hospital Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Tel: 00 32 (0)16.34.62.85, Fax: 00 32 (0)16.34.69.01, e-mail: hans.prenen@uz.kuleuven.ac.be

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Materials and Methods

Cell culture. The GIST882 cell line, that carries a homozygous K642E missense *KIT* mutation, was a kind gift from Dr. Jonathan Fletcher (12). GIST882 cells were grown in culture flasks in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Reagents. Imatinib mesylate was provided by Novartis Pharma (Basel, Switzerland) and dissolved in 100% DMSO at 10 mM stock for *in vitro* studies. For oral administration in animal experiments, imatinib was dissolved in distilled sterile water. Rabbit polyclonal antibody for MAPK and phospho-KIT (Y703) were from Zymed Laboratories (San Francisco, CA, USA); rabbit polyclonal anti-KIT and anti-Ki-67 were from DAKO (Glostrup, Denmark); rabbit polyclonal antibodies for phospho-MAPK (Thr 202/204), phospho-AKT and AKT were from Cell Signalling (Beverly, MA, USA); goat monoclonal antibody for protein kinase C theta (PKCθ) was from Santa Cruz (Santa Cruz, CA, USA).

Animals. Female adult athymic nude mice (NMRI *Nu/nu*), with an average body weight of 30 g, were used. The animals were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12-hour light/12-hour dark schedule and fed autoclaved chow and water *ad libitum*. The animal studies had been approved by the Animal Ethics committee of the Catholic University Leuven, Belgium.

Subcutaneous implantation of GIST882 tumour cells and drug administration. For inoculation into nude mice, GIST882 cells were washed in phosphate-buffered saline (PBS), digested with trypsin, re-suspended in DMEM containing foetal bovine serum and pooled. After centrifugation, the cells were re-suspended in PBS at a density of 3x10⁶ viable cells per 100 µL. The cell suspension (100 µL) was injected subcutaneously in the right and left flanks of 15 athymic Nu/Nu mice. Tumour growth was assessed every 3 days. Three orthogonal diameters were measured with a vernier calliper and used to calculate the volume of the tumour using the formula $a \times b \times c \times \pi/6$ (13). When the tumours had reached a volume of about 500 mm³, the mice were randomised into 3 groups (10 tumours per group = 5 mice).

The first 2 groups received imatinib mesylate by gavages (150 mg/kg twice daily), while the third control group only received vehicle (sterile water). The tumour volume and body weight were recorded every 2 to 3 days. The mice of group 1 (imatinib-treated) and group 3 (control) were sacrificed on day 8 of treatment and the tumours were resected for further analysis. For histological and immunohistochemical staining procedures, one part of the tumour tissue was fixed in formalin and embedded in paraffin. Another part of the tumour was snap-frozen and stored in liquid nitrogen for Western analysis. Mice of group 2 were treated with imatinib for 20 days to evaluate the effect on tumour growth of a long duration of treatment.

Immunohistochemical staining for KIT (CD-117) and Ki-67. Histopathological examination was performed on tissue fixed in 6% buffered formalin and embedded in paraffin. Sections (5 µm) were cut from the paraffin blocks for routine haematoxylin and eosin

(H&E) and immunohistochemical staining (avidin-biotin-peroxidase complex method) using the polyclonal rabbit anti-human antibody against CD117 (dilution 1:250, DAKO) and Ki-67 antibody (dilution 1:250). The CD117 immunostaining was performed with and without antigen retrieval (20 minutes microwave in citrate buffer pH6).

Western analysis. Snap-frozen tumour specimens were lysed in ice-cold lysis buffer containing 1% Triton X 100, 1 mM Na₂EDTA, 50 mM NaF, 5 mM Na₃VO₄, 20 µM PAO and supplemented with complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany). The tissues were homogenised using a dounce homogeniser (Fischer Bioblock Scientific, Illkirch, France). The lysates were rocked for 30 minutes at +4°C and then centrifuged at 21,000 xg for 30 minutes +4°C. The supernatants were removed to a fresh tube and the protein content was determined using Bradford reagent (Bio-Rad). Thirty micrograms of proteins were separated on SDS-polyacrylamide gels and blotted to PVDF membranes (Amersham Pharmacia Biotechnology, UK). The membranes were blocked in PBS containing 5% blocking reagent (dried milk) and incubated with a primary antibody. The membranes were then washed in PBST, probed with anti-rabbit immunoglobulin-HRP conjugate and incubated with ECL substrate (Pierce).

Small animal PET imaging studies. GIST882 tumour-bearing mice were imaged using a Focus micro-PET[®] scanner (Concorde Microsystems Inc., Knoxville, TN, USA) using [¹⁸F]-fluoro-deoxyglucose (FDG) to image the glucose metabolism of the tumour. PET-scans were performed before treatment (day 0), 24 hours after treatment (day 1) and after 1 week of treatment with imatinib (day 8). The mice were anaesthetized with Isofluran and 300 µCi of FDG was administered by tail vein injection. Before injection with FDG, the mice were restricted from food for at least 6 hours. One hour after the injection, 10-minute static scans were performed. Images were reconstructed using filtered back-projection with a RAMP 0.5 filter. The images were displayed in AMIDE and 3-dimensional regions of interest (ROIs) were drawn over the tumour and the right liver lobe (volume=0.52 ml). The tumour volume was fixed to the calliper measured volume. The mean radioactivity in the ROI's was converted to percent of the injected dose per gram of tissue (%ID/g) by a calibrated cylinder factor and by dividing with the injected dose (corrected for residual and decay). A tumour to liver ratio (T/L) was determined by dividing the mean tumour %ID/g by the mean liver %ID/g.

Statistical analysis. The results are expressed as mean ± standard error of the mean (SEM). The small animal PET measurements were analysed with a paired Student's *t*-test.

Results

Tumour establishment and growth inhibition after treatment with imatinib. Gastrointestinal stromal tumours were established by subcutaneous injection of 3x10⁶ GIST882 cells into the right and left flanks of 15 nude mice. All 15 mice developed bilateral tumours 4 weeks after injection. When the tumours had reached a mean volume of about 500 mm³ (10 weeks after injection), the mice were randomised into

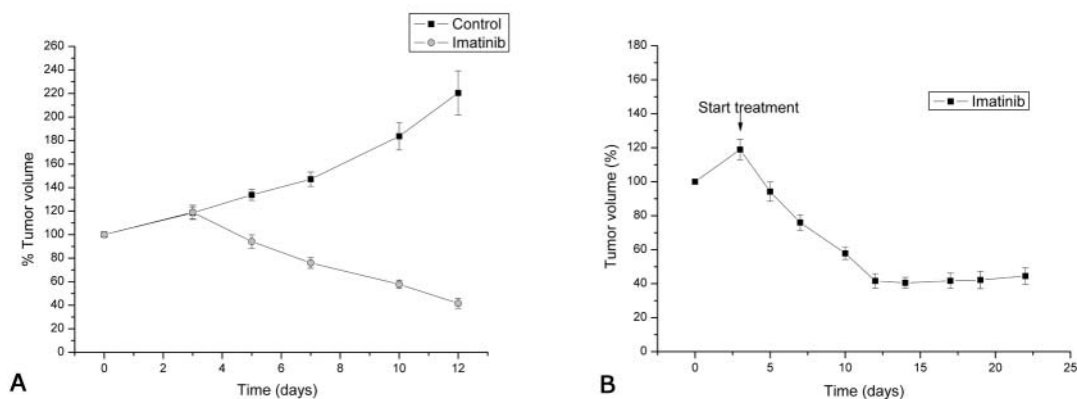


Figure 1. Effect of imatinib on the growth of GIST882 tumour xenografts in nude mice. Nude mice bearing GIST882 tumor xenografts were treated daily with placebo (vehicle) or imatinib at 150 mg/kg twice daily. The percentage of tumour volume was calculated by dividing the volume at each time-point with the average volume of the tumours at the time when the treatment began. A significant decrease of tumour volume was seen after 1 week of treatment (A). When treated for 20 days there was no further decrease of the tumour volumes in comparison with 1 week of treatment, but there still was an inhibition of tumour growth as shown in (B).

3 groups. The mean body weight and tumour volume did not differ different between the 3 groups. The first 2 groups received imatinib mesylate by gavages, the third control group only received vehicle (sterile water). As shown in Figure 1A, the size of the GIST882 tumour xenografts increased proportionally in the control group treated only with the vehicle. Treatment with imatinib mesylate at a concentration of 150 mg/kg body weight twice daily completely suppressed the growth of the tumours. After 1 week of treatment, the tumour volumes even decreased by 60%. Mice treated with imatinib did not show severe signs of morbidity during the treatment, only a mild skin rash was observed in the imatinib-treated mice. The body weight was not affected by the treatment either. The second group of mice was treated for 20 days with imatinib twice daily (Figure 1B). After an initial decrease in tumour volume during 1 week of treatment, there was no further volume decrease, but still an inhibition of tumour growth until 20 days of treatment with imatinib.

Evaluation of response to imatinib by non-invasive in vivo tumour imaging with high resolution small animal positron emission tomography. A total of 10 mice were imaged (5 in the control group and 5 in the imatinib-treated group). The T/L ratio in the imatinib-treated group was 0.575 ± 0.067 on day 0, decreased by 31.6% to 0.394 ± 0.027 on day 1 ($p < 0.05$) and increased again to 0.630 ± 0.034 on day 8 (Figure 2). In the control group, the values were 0.927 ± 0.133 on day 0, 0.843 ± 0.126 on day 1 and 0.816 ± 0.107 on day 8, differences which were not statistically significant (Figure 2A). An example of FDG uptake in a nude mouse before treatment and 24 hours after the start of treatment with imatinib twice daily is provided in Figure 2B.

Histology and immunohistochemistry. After 1 week of treatment with imatinib mesylate (group 1) or with vehicle only (group 3), the GIST882 tumours were resected. Routine H&E stains of the tumours revealed a uniform population of spindle cells (Figure 3A). After 7 days of treatment, the morphology, as visualised by H&E staining, was not different between the imatinib-treated and the control groups. Although the cultured GIST882 cells clearly expressed KIT, it was not detected by immunohistochemistry in either group of GIST tumours (Figure 3B), suggesting that the KIT expression of GIST xenografts growing in nude mice can decrease with time and become undetectable by immunohistochemistry.

The tumours were also evaluated for the presence of Ki-67. Ki-67 is a nuclear protein that is linked to the cell cycle and is a marker of cell proliferation. Ki-67 staining was completely negative in the imatinib-treated tumours in contrast to the controls (Figure 3C, D).

Evaluation of KIT signalling pathways by Western blot. Another part of the resected GIST882 xenografts was used for Western analysis (Figure 4). All xenografts had demonstrable KIT expression. The GIST882 cell line was used as a positive control. The upper and lower bands in the total KIT stain are mature (145 kDa) and immature (125 kDa) forms, respectively. In tumours treated with imatinib, a reduction of the 125 kDa form was observed. KIT phosphorylation varied from tumour to tumour and was completely inhibited by imatinib treatment. The downstream KIT signalling intermediates, MAPK and AKT, were activated in GIST882 xenografts and inhibited by imatinib treatment. Notably, AKT phosphorylation increased in GIST882 xenografts, while MAPK

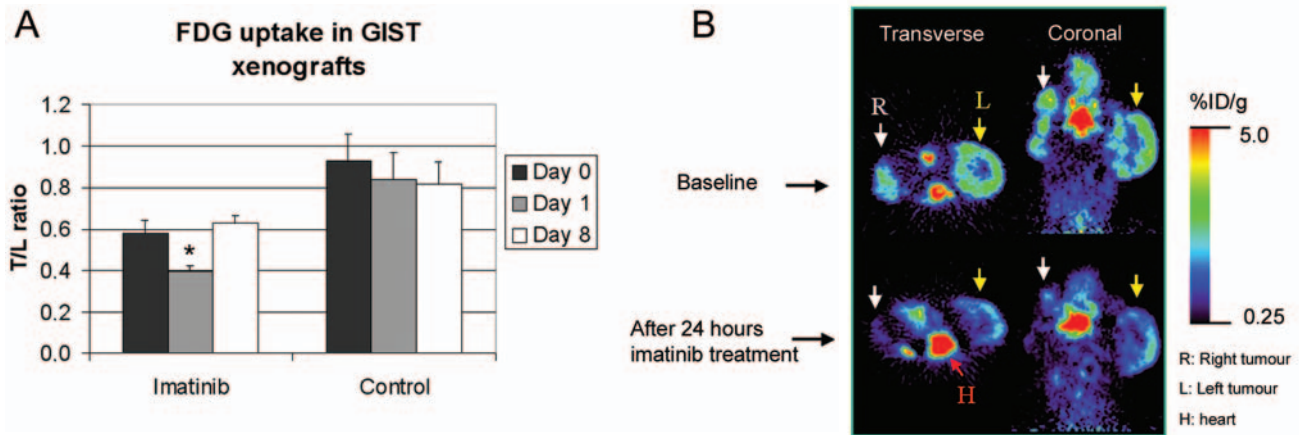


Figure 2. A. Influence of imatinib treatment on the FDG uptake in GIST xenografts. The mean tumour to liver ratio is given for day 0 and days 1 and 8 after imatinib treatment. The decrease on day 1 in the imatinib group was significant. The error bar represents the standard error of the mean. * $p < 0.05$; B. Example of FDG uptake in a nude mouse before and 24 h after treatment with imatinib.

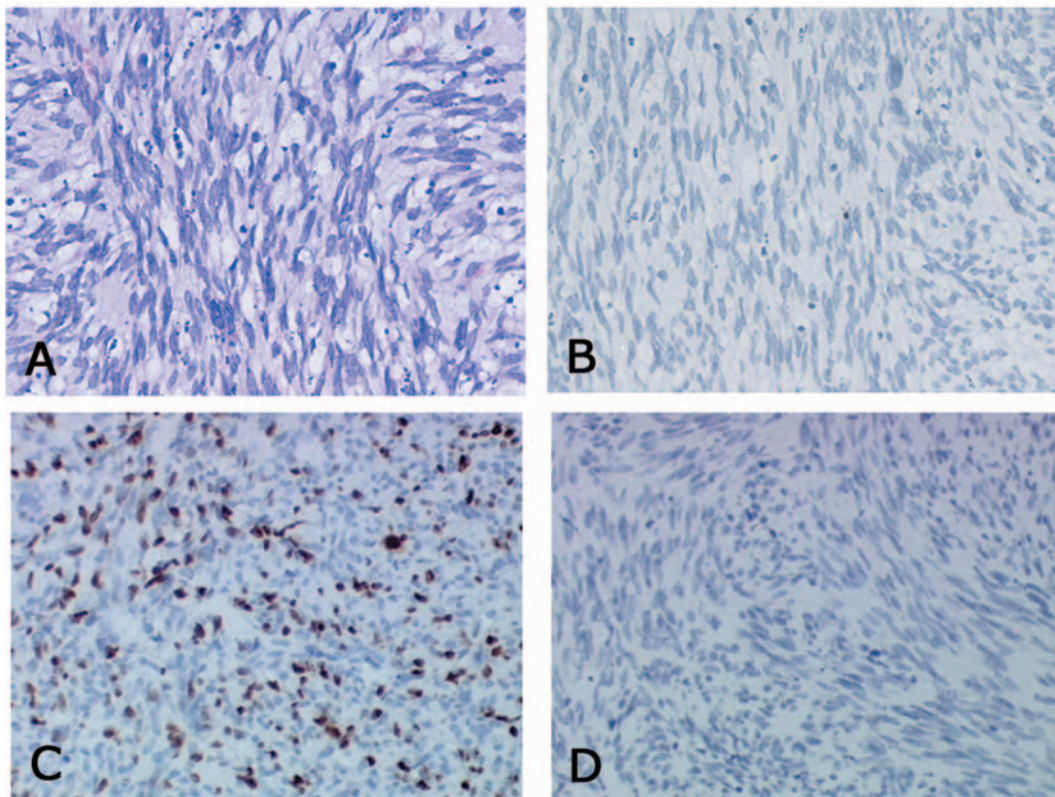


Figure 3. Haematoxylin and eosin staining of GIST882 tumours growing in nude mice showed a uniform population of spindle cells (A). Immunohistochemical detection of KIT was negative in all tumours (B). Ki-67 staining was clearly positive in the untreated tumours (C) and negative in the imatinib-treated ones (D). Original magnification $\times 40$.

phosphorylation was relatively low in comparison with the GIST882 cells grown *in vitro*. Next PKC θ , which is highly expressed in GISTs, was evaluated (14). Our study showed

that the level of PKC θ decreased in the imatinib-treated xenografts in comparison with the control xenografts (Figure 4).

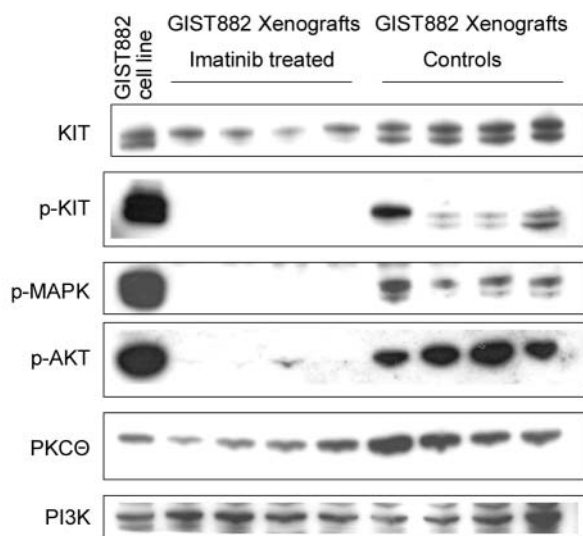


Figure 4. Expression of total KIT, p-KIT, p-AKT, p-MAPK, PKC θ and PI3K in GIST882 xenografts, untreated (control) and treated with imatinib. PI3K was used to confirm equal protein loading.

Discussion

The tyrosine kinase inhibitor imatinib mesylate has proven to be highly effective for the treatment of inoperable and/or metastatic GIST, a tumour known to be very chemo- and radiotherapy resistant. Despite the good clinical results, GISTs can become resistant to imatinib over time (11). Hence, alternative therapeutic strategies are needed after failure of imatinib. To study new targeted therapies in GIST, we established a GIST mouse model by injecting GIST882 cells into the flank of nude mice. The present study is the first report on a subcutaneous GIST mouse model. To analyse the effect of imatinib treatment on GIST882 xenografts, the mice were orally treated by gavages with imatinib twice daily. Initially, shrinkage of the tumour was seen during 1 week of treatment, followed by stabilisation of the tumour volumes until 20 days of treatment.

Tumour-bearing mice were also analysed through imaging by micro positron emission tomography (PET) employing ^{18}F -FDG as a radioactive tracer. In patients, imaging by PET-scan was very effective for the early prediction of response in GISTs treated with imatinib (15). GIST xenografts showed a relatively low FDG up-take with a tumour to liver ratio ranging from 0.41 to 1.29. A significant decrease of 31.6% in FDG up-take was seen in GIST tumours in mice treated with imatinib for 24 hours, which correlates with the initial macroscopic response of the GIST xenografts to imatinib. After 1 week of treatment, FDG up-take increased and returned to baseline levels. The

increase of FDG up-take could be attributed to the sustained proliferation of imatinib-resistant cells within the tumours. However, this finding is in contrast with the negative staining of the proliferation marker Ki-67 found by immunohistochemical analysis after 1 week of treatment. We recently showed that a rapid decline in glucose uptake following imatinib treatment in GIST cells was dependent on glucose transporter impaired anchorage to the plasma membrane (16). It might be possible that GIST cells can develop other mechanisms in order to regain their glucose influx, which is an important source of energy necessary for cell survival. The basis of the observed phenomena requires further investigation.

Resected tumours were also used for Western analysis. Although KIT protein was present, as shown by Western analysis, it could not be detected by immunohistochemical staining, most probably because the amount of KIT protein was relatively low. Subsequently, signalling pathways were analysed by Western analysis. Phosphorylated KIT, MAPK and AKT were completely inhibited in the imatinib-treated xenografts in comparison with the controls. Recent studies have shown that PKC θ is highly expressed in GISTs and not in other mesenchymal tumours (14, 17). Determination of the expression of PKC θ by Western analysis revealed a decreased expression in the imatinib-treated tumours, indicating the close association with the activated form of the KIT protein.

In conclusion, this model represents a valuable tool to study targeted therapies in GISTs and will be useful to study novel strategies to overcome imatinib resistance. Non-invasive assessment of glucose metabolism with FDG small animal PET is of particular value as it allows monitoring of each animal at all time-points, thereby reducing the number of experimental animals. As FDG-PET is used to monitor clinical therapies, these results can be correlated to clinical data and, as such, accelerate translational research.

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