

Multimodal imaging of tumor response to sorafenib combined with radiation therapy: comparison between diffusion-weighted MRI, choline spectroscopy and ^{18}F -FLT PET imaging

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The purpose of this study was to determine the value of different imaging modalities, that is, magnetic resonance imaging/spectroscopy (MRI/MRS) and positron emission tomography (PET), to assess early tumor response to sorafenib with or without radiotherapy. Diffusion-weighted (DW)-MRI, choline ^1H MRS at 11.7 T, and ^{18}F -FLT PET imaging were used to image fibrosarcoma (FSa1) tumor-bearing mice over time. The imaging markers were compared with apoptosis cell death and cell proliferation measurements assessed by histology. Anti-proliferative effects of sorafenib were evidenced by ^1H MRS and ^{18}F -FLT PET after 2 days of treatment with sorafenib, with no additional effect of the combination with radiation therapy, results that are in agreement with Ki67 staining. Apparent diffusion coefficient calculated using DW-MRI was not modified after 2 days of treatment with sorafenib, but showed significant increase 24 h after 2 days of sorafenib treatment combined with consecutive irradiation. The three imaging markers were able to show early tumor response as soon as 24 h after treatment initiation, with choline MRS and ^{18}F -FLT being sensitive to sorafenib in monotherapy as well as in combined therapy with irradiation, whereas DW-MRI was only sensitive to the combination of sorafenib with radiotherapy. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: sorafenib; radiotherapy; DW-MRI; choline spectroscopy; ^{18}F -FLT PET imaging

1. INTRODUCTION

The predictive value of markers of response to treatment is of crucial importance in the management of cancer patients by allowing better individualization of treatment. In the clinical setting, the prediction of tumor response to therapy is usually determined from measurements of tumor size evidenced by morphological imaging (1). However, tumor shrinkage can take several weeks to manifest. Therefore, early indication of tumor treatment response using imaging biomarkers has become a major area of research. For example, the 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG) uptake in treatment-sensitive tumors by positron emission tomography is used as a marker of disease progression in some clinical applications (2).

In the MR field, diffusion-weighted magnetic resonance imaging (DW-MRI) assesses the diffusion of water molecules, the quantified apparent diffusion coefficient (ADC) being correlated with cellular density, presence of necrosis and, in the therapy setting, tumour cell apoptosis and proliferation indices (3–6). Magnetic resonance spectroscopy (MRS) detects changes in metabolites. Activated choline metabolism, which is characterized by increased phosphocholine (PCho) and total choline-containing compounds (tCho) have been associated with aggressiveness in breast cancer (7). Because of the ability of MR spectroscopy to detect changes in choline metabolism, the method is being exploited for diagnosis,

prognosis, monitoring of response and the development of novel therapies (8). A particular advantage of ^1H MR choline spectroscopy and DW-MRI is that it does not require intravenous contrast media, thus enabling its repeated use in patients (9).

Finally, recent human clinical trials reports have assessed 3'-deoxy-3'- ^{18}F fluorothymidine (^{18}F -FLT) used in conjunction with positron emission tomography (^{18}F -FLT PET), as a proliferation imaging biomarker for prediction of tumour response to treatment (10,11). Cell proliferation being increased with malignant

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transformation, the use of thymidine analogs provides a useful way of targeting DNA replication (12). More recently, both dynamic nuclear polarization (DNP) using hyperpolarized [1,4- $^{13}\text{C}_2$]fumarate (13) and micro-PET imaging using a caspase-3/7-specific ^{18}F -labeled isatin sulphonamide (14), have shown promising properties in detecting cell death in the pre-clinical setting in tumor models.

We previously described that 2 days of treatment with sorafenib (Nexavar) was able to induce a reoxygenation window in two distinct tumor models, which could be exploited for improving the radiation response of experimental tumors (15). Sorafenib was the first RAF kinase inhibitor to enter human clinical testing and is approved for use in renal cell and hepatocellular carcinoma (16). In our study, the major focus was the increase in tumor oxygenation that was shown to be the result of two factors: (i) an increase in blood flow, which might be linked to the anti-angiogenic effect of sorafenib (vascular normalization); and (ii) a decrease in oxygen consumption, owing to an alteration of the mitochondrial activity (15). The aim of the current study was to assess DW-MRI, ^1H MR spectroscopy and ^{18}F -FLT PET imaging as early biomarkers of response after treatment with sorafenib alone or combined with radiation therapy.

2. RESULTS AND DISCUSSION

2.1. Diffusion-weighted MRI

We did not observe any significant modification of ADCw (Apparent diffusion coefficient of water) value after 2 days of treatment with sorafenib ($-16 \pm 9\%$) or DMSO ($-20.7 \pm 8.7\%$; day 0 vs day 2). However, we observed a significant increase in ADCw value 24 h after irradiation in the sorafenib treated group ($+41 \pm 20.8\%$; day 2 vs day 3) while ADCw values in the control group remained unchanged 24 h after irradiation ($-28.1 \pm 9.6\%$; day 2 vs day 3; Fig. 1).

It has been described that, soon after initiation of therapy, transient decreases in ADC can be observed, related to cellular swelling or to extracellular space that may be mediated by vascular normalization if anti-angiogenic drugs are given (3). It has been shown that anti-vascular endothelial growth factor therapies in brain tumors lead to an initial reduction in vasogenic edema that lowers ADC values (17). Sorafenib, in the early phase, has been shown to temporarily decrease ADC value followed by an increase in patients with advanced hepatocellular carcinoma (18). These observations indicate that ADC changes are dependent on complex interplays of biophysical processes (3). However, regarding our data, sorafenib, which is also an anti-angiogenic drug, does not show significant reduction or increase in ADCw after 2 days of treatment, whereas, if we compare with TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay results, we observe a significant increase of apoptosis at day 2, so we hypothesize that DW-MRI is not sensitive enough to show the effect of sorafenib alone, but is adequate to show the effects of the combination of sorafenib and radiation therapy.

2.2. ^1H MR Choline Spectroscopy

The choline peak areas were normalized to the corresponding unsuppressed water signal. We observed a significant decrease of the total choline peak after 2 days of treatment with sorafenib

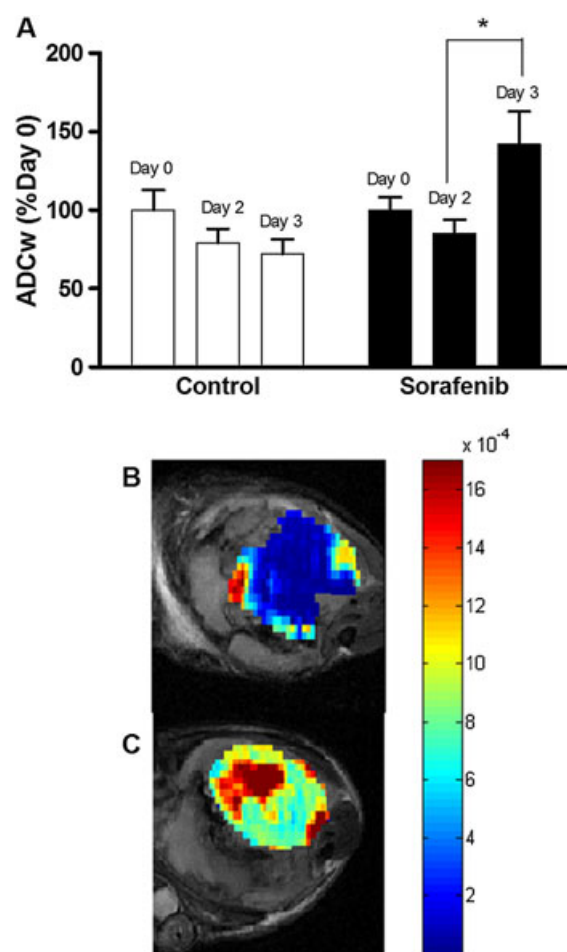


Figure 1. Evolution of the tumor ADCw value expressed as percentage of the ADCw obtained for mice at day 0. Note the significant increase ($p < 0.05$) between day 2 and day 3 in the sorafenib group (A). ADCw maps of tumor-bearing mice before (day 2; B) and 24 h after irradiation (day 3) (C) of the sorafenib-treated group. Each image represents an axial DW image (b -value=0) of the mouse leg with the tumor area transformed as an ADCw map.

($n=6$; $-49.3 \pm 9.7\%$; day 0 vs day 2), but no further modification owing to irradiation combined with sorafenib (day 2 vs day 3). The total choline peak was not significantly different in the control group ($n=5$; $-4.3 \pm 9.4\%$) between the pre-treatment (day 0) and the post-treatment (day 2) spectrum and after irradiation (day 3) in the control group ($-32.5 \pm 27.3\%$; Fig. 2).

Measurement of tCho signal using ^1H MR choline spectroscopy to assess responses to treatment has been reported and useful for various preclinical and clinical studies (19–22). More specifically, U0126, a MAPK inhibitor has shown a drop in phosphocholine (PCho) in different tumor cell lines by inhibition of choline uptake and phosphorylation following MAPK signaling blockade (23–25). Recent studies indicate that inhibition of the MAPK signaling pathway resulted in reduced PCho and tCho levels and that this effect is primarily mediated by choline kinase inhibition as a result of its reduced expression downstream of Hypoxia Inducible Factor 1 (HIF1) activation (8,26). Indeed HIF1 is activated under hypoxic conditions and degraded under normoxic conditions. A close correlation was observed between region of high tCho levels and hypoxia *in vivo* owing to the regulation of choline kinase by HIF1 (26). It would be interesting

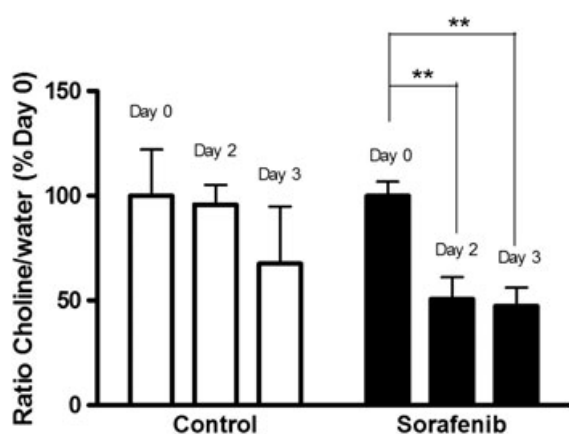


Figure 2. Evolution of the choline peak before and two days after tumor treatment (Control and Sorafenib groups) and 24 hours after combined treatment with radiation. The choline peak area is expressed as a choline to water ratio and as percentage of the ratio obtained at day 0.

to see whether tumor reoxygenation induced by 2 days of treatment with sorafenib (15) would be able to inhibit HIF1 and thereby decrease choline kinase expression and so tCho signal.

2.3. ^{18}F -FLT PET Imaging

We observed a significant decrease ($p < 0.05$) in the ^{18}F -FLT uptake (ratio T/B) after 2 days of treatment with sorafenib ($n = 4$; $-35.9 \pm 11.9\%$; day 0 vs day 2) and 24 h after irradiation combined with 2 days of treatment with sorafenib ($-45.1 \pm 10.2\%$; day 0 vs day 3; $p < 0.01$), but no difference before and after irradiation (day 2 vs day 3). However we did not observe any significant decrease in ^{18}F -FLT uptake between day 0 and day 2 ($+9.5 \pm 10.7\%$) and between day 0 and day 3 ($+0.9 \pm 9.9\%$) in the control group ($n = 7$) (Fig. 3).

In the clinic the effect of sorafenib on ^{18}F -FDG uptake has been explored with reduction in SUVmax (The Standardized Uptake Value maximum) after 3 weeks, in accordance with a partial response on CT scan in week 12 (27). ^{18}F -FLT has also been found to predict response and patient outcome to EGFR (Epidermal Growth Factor Receptor) inhibitors with a reduction in SUVmax of 410.4% at 7 days post gefitinib therapy predicting RECIST (Response Evaluation Criteria In Solid Tumors) response on CT at 6 weeks (28,29). Another inhibitor of the same MAPK pathway, PD0325901, has been shown to decrease ^{18}F -FLT uptake in an experimental tumor and it was correlated to a decrease of cell proliferation (30). Thymidine kinase 1 (TK1) is expressed primarily in S phase and is thus highly expressed in proliferating cells, but is expressed at low levels in quiescent cells. TK1 catalyzes the phosphorylation of ^{18}F -FLT to ^{18}F -FLT-monophosphate, which, because of its negative charge, is trapped in cells (29). Therefore, sorafenib, which selectively arrests tumor cells in G1 and decrease S phase (31–33), would be predicted to cause a decrease in tumor ^{18}F -FLT uptake and tracer retention. Our results corroborate the fact that ^{18}F -FLT-PET is a sensitive imaging biomarker for detecting the anti-proliferative effect of sorafenib.

2.4. Histology

TUNEL staining of untreated ($0.6 \pm 0.3\%$) and sorafenib treated tumors ($3.6 \pm 0.6\%$) shows an increase in positively stained cells after 2 days of treatment ($p < 0.05$) which was not observed in

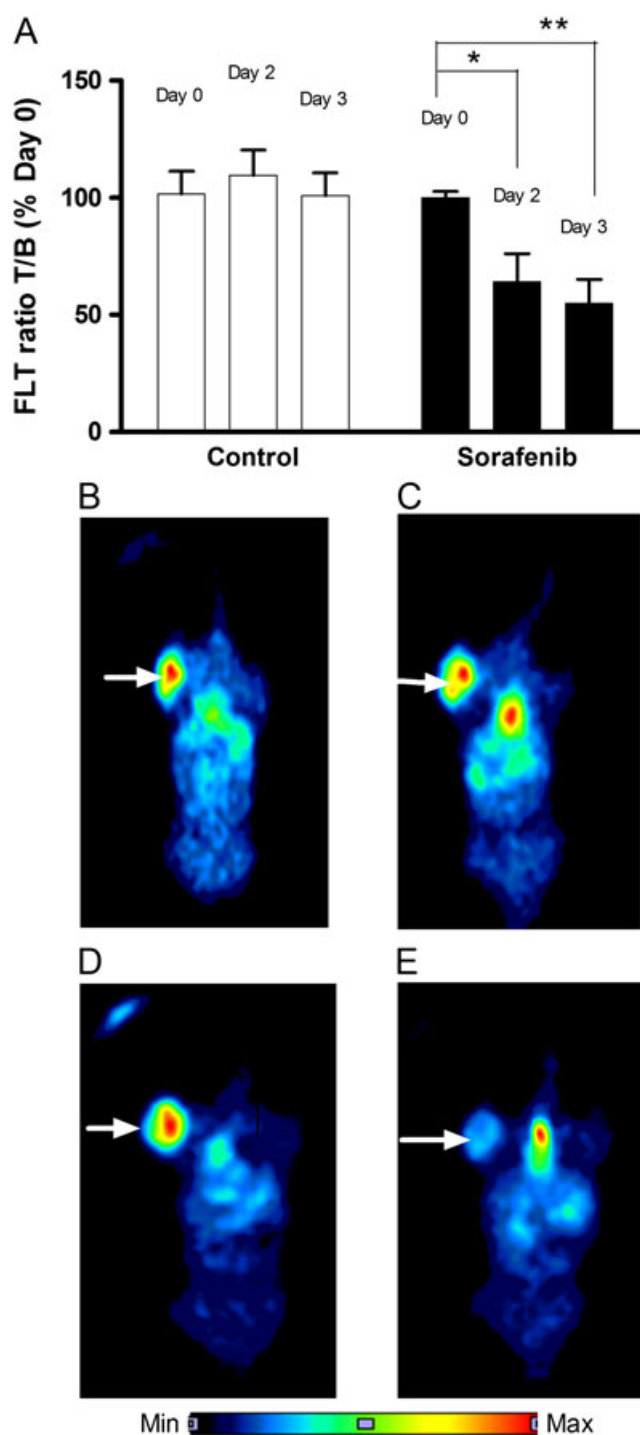


Figure 3. Evolution of the ^{18}F -FLT PET uptake expressed in T/B ratio (A) for sorafenib and control groups. Representative images of ^{18}F -FLT PET imaging in the control group at day 0 (B) and day 2 (C), and in the sorafenib group before (D) and after 2 days of treatment (E). Images were normalized.

DW-MRI. However in agreement with the ADCw value obtained using DW-MRI, we observed a more significant increase after sorafenib combined with irradiation ($6.6 \pm 1\%$; $p < 0.001$), while we did not observe any significant increase after irradiation alone ($1.3 \pm 0.3\%$) compared with the untreated group. Regarding Ki67 staining (quantification of proliferation), we observed a significant decrease ($p < 0.05$) between untreated

($74.6 \pm 7.1\%$) and sorafenib-treated tumors ($45.8 \pm 7.4\%$) and sorafenib combined with irradiation ($39.3 \pm 6\%$), but no additional effect was observed owing to irradiation. Control irradiated tumors ($60 \pm 4.5\%$) did not show significant difference with untreated tumors (Fig. 4). In our study, changes in total choline signal and ^{18}F -FLT results are in accordance with the anti-proliferative effect of sorafenib as measured using histological Ki67 staining.

2.5. Tumor Growth Delay

The impact of sorafenib on fibrosarcoma (FSall) tumors growth was compared with tumor growth of control mice. As shown in Fig. 5, sorafenib was able to slow down the tumor growth at the dose of 45 mg kg^{-1} per day during 6 days, compared with the control group. Sorafenib significantly affected tumor growth, as the times to reach 12 mm in tumor diameter were 7.5 ± 0.47 days for the control group and 12 ± 1.2 days for the sorafenib-treated group ($p < 0.01$). [Data shown for sorafenib + X-rays come from a previous study (15) to underline the impact of combined sorafenib treatment with X-rays on tumor growth.]

3. CONCLUSIONS

In this study, MRI, MRS and PET imaging modalities were assessed to monitor early tumor response to sorafenib combined or not to radiation therapy. We previously showed a radiosensitizing effect of sorafenib in FSall tumors owing to increased oxygenation (15). In the current study, DW-MRI, ^1H MR choline spectroscopy and FLT PET imaging were performed before treatment (day 0), after 2 days of treatment with sorafenib (day 2) and 24 h after irradiation consecutive to 2 days of treatment with sorafenib (day 3).

In conclusion, we observed similar patterns for ^1H MR spectroscopy and ^{18}F -FLT PET imaging, which are described to reflect cell proliferation. This was corroborated by studying the anti-proliferative effect of sorafenib using Ki67 staining. Nevertheless, these two imaging markers were not able to identify the effect of the combination of sorafenib with radiation therapy. On the contrary, ADC values did not change after sorafenib treatment alone (contrary to apoptosis measurement via the TUNEL assay), but were sensitive to sorafenib combined with radiotherapy, a result that is in agreement with the TUNEL assay. DW-imaging

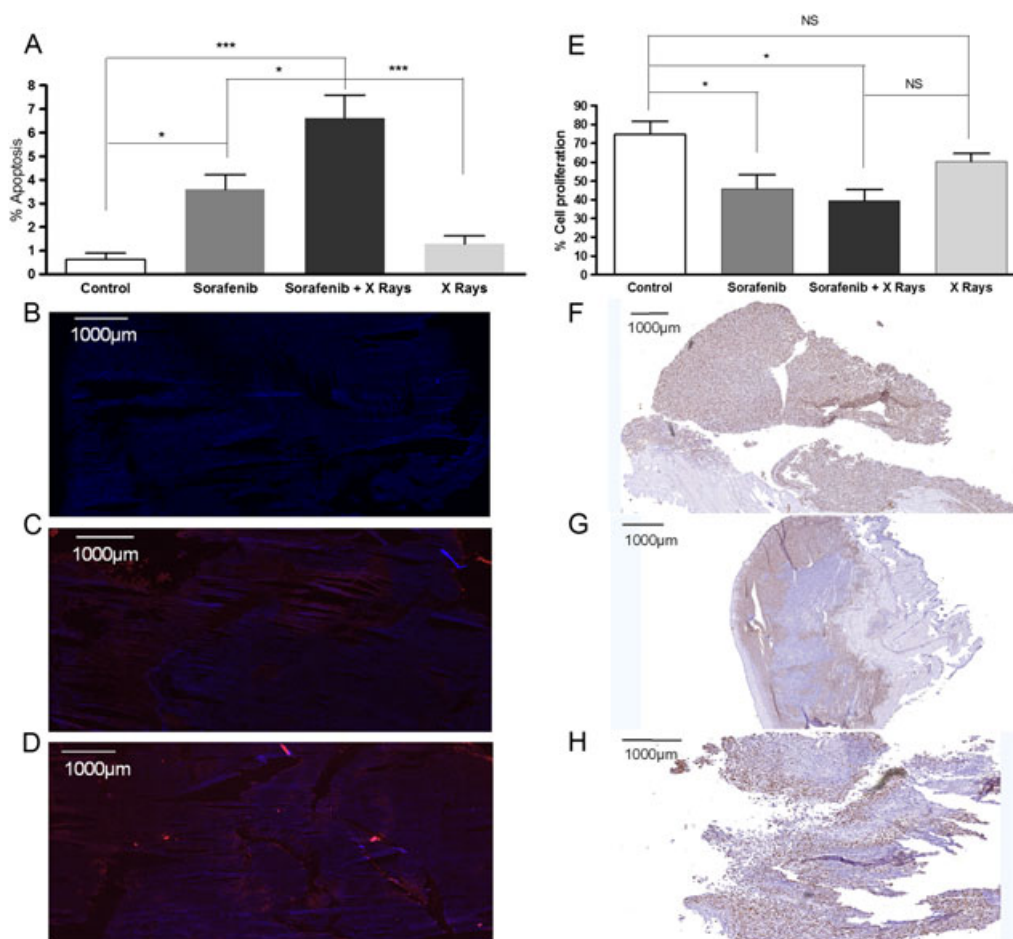


Figure 4. Quantification of apoptosis cell death using TUNEL assay (A) and cell proliferation with Ki 67 immunohistological staining (E). Representative histological staining performed on tumor slices. Fluorescent TUNEL stained section before treatment (B) and from tumors 2 days after treatment with sorafenib (C), and 24 hours after sorafenib combined with radiation (D). TUNEL-positive nuclei are stained in red and the nuclear counterstain 4,6-diamidino-2-phenylindole in blue. Ki 67 stained section from untreated mice (F) and from mice 2 days after treatment with sorafenib (G), and 24 hours after sorafenib combined with radiation (H). Ki 67 positive nuclei are stained in brown. Only tumor regions were used for quantification; the surrounding muscle tissue was not taken into account.

DMSO 6 days	Sorafenib 6 days		Sorafenib 2 days+ X rays
7,5 ± 0,47	12 ± 1,2		23,4 ± 1,8

Figure 5. Tumor growth of mice with FSall tumors treated with Sorafenib or DMSO. The treatment was injected at a tumor size of 8 ± 0.5 mm ($n=8$ per group). Data are presented as the time in day to reach 12 mm in tumor diameter. [Data shown for sorafenib + X-rays come from a previous study (15) to underline the impact of combined sorafenib treatment with X-rays on tumor growth.]

is, however, not described to probe any particular type of cell death and therefore such comparison has to be taken with caution. The three imaging markers were able to show early tumor response as soon as 24 h after treatment initiation, with choline MRS and ^{18}F -FLT being sensitive to sorafenib in monotherapy as well as in combined therapy with irradiation, whereas DW-MRI was only sensitive to the combination of sorafenib with radiotherapy.

An undesired effect of anti-angiogenics in their normalization phase could be an increase in cell proliferation resulting from a transient enhancement of blood perfusion, oxygenation and nutrient supply (34). The current study indicates that proliferation is not increased and shows the anti-proliferative effect of the anti-angiogenic drug sorafenib in experimental tumors, probably owing to sorafenib impact on endothelial cells but also on tumor cells by inhibiting the MAPK pathway (15).

4. EXPERIMENTAL

4.1. Animal and Tumor Models

FSall fibrosarcoma cells (35) were inoculated in the legs of C3H/HeOulco mice. For inoculation, 10^6 cells in 0.1 ml of medium were injected intramuscularly. Tumors were allowed to grow up to 8 mm in diameter prior to experimentation. Animals were anesthetized by inhalation of isoflurane (St Luc Hospital, Brussels, Belgium) mixed with 21% oxygen (air). All animal experiments were conducted in accordance with national and university animal care regulations.

4.2. Treatments and Protocol

Sorafenib (LC Laboratories, MA, USA) dissolved in DMSO (Dimethyl sulfoxide) (Invitrogen), was delivered intraperitoneally at a dose of 45 mg kg^{-1} via 100 μl injections. Tumors were imaged at day 0 (before injection of sorafenib or vehicle), at day 2 (after 2 days of injection with sorafenib or vehicle) and finally at day 3 (24 h after 2 days of treatment with sorafenib or vehicle followed by irradiation with 20 Gy of X-rays). The protocol is illustrated on Fig. 6. The same mice were monitored from day 0 to day 3 for one imaging marker, but different batches of mice were used for DW-MRI [control ($n=5$) and sorafenib ($n=4$)], ^1H MR spectroscopy [control ($n=6$) and sorafenib ($n=5$)], ^{18}F -FLT PET [control ($n=7$) and sorafenib

($n=4$)], histology ($n=4$ per group) and growth delay ($n=8$ per group).

4.3. MR Studies

Animal experiments were performed with an 11.7T system (Bruker, Biospec), and with a quadrature volume coil (inner diameter of 40 mm). The animals were anesthetized by isoflurane inhalation (3% in air for initiation and 1–2% in air for maintenance) and their hind legs were fixed in clay. Animals were laid on a warm waterbed to maintain body temperature using a rectal temperature probe and a pressure cushion was placed near the chest to monitor respiration.

4.4. Diffusion-weighted MRI

For DW-MRI, a transverse echo planar imaging sequence was used with the following acquisition parameters: repetition time (TR)/echo time (TE), 3000/27.96 ms; duration of diffusion gradients (d), 7 ms; separation of diffusion gradients (Δ), 14 ms; slice number, 6; slice thickness, 1 mm; interslice distance, 1.5 mm; in-plane resolution, 0.234 mm^2 per pixel; acquisition time, 4 min 12 s. DW images were acquired using b -values of 0, 200, 400, 600, 800 and 1000 s mm^{-2} . The b -value is equal to $g^2 \cdot Gd^2 \cdot d^2 [\Delta - (d/3)]$, where Gd is the strength of the diffusion weighting gradient and g is the gyromagnetic ratio for protons. Mean apparent diffusion coefficients (ADC_w) were calculated from the DW images and averaged for every slice where the tumor was found using a homemade program in Matlab software (The MathWorks Inc., Natick, MA, USA) to define regions of interest (ROI). The exponential decay of signal as a function of the b -value was measured according to the Stejskal–Tanner equation, $S = S_0 e^{-b \cdot \text{ADC}_w}$, where S_0 is the signal intensity achieved without gradient and S is the signal intensity with diffusion weighting.

4.5. ^1H MR Choline Spectroscopy

For single voxel spectroscopic data acquisition, voxel localization was performed on the anatomical images obtained by the 'tripilote' and turbo RARE sequences. Field homogeneity was optimized over the selected volume of interest by shimming. We obtained the water signal (PRESS) Point Resolved Spectroscopy for each tumor; the shim was acceptable if the line width was <50 Hz. Automatic shimming and manual water suppression (VAPOR) were used. Proton MR spectra were acquired using a PRESS sequence (TR/TE, 2500/20 ms). Voxel size of $4 \times 4 \times 4 \text{ mm}^3$ and 200 averages were used for MRS studies in all tumors. Acquisition time for spectroscopic studies was about 8.5 min. MR spectra were analyzed on an independent workstation using jMRUI software version 5.0.

The metabolite model signals used in QUEST (quantitation based on quantum estimation) were simulated in NMR-SCOPE (NMR spectra calculation using operators; jMRUI). Phase-

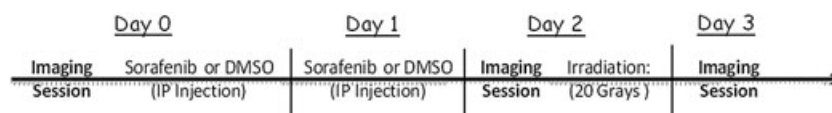


Figure 6. Schematic representation of the experimental set up described in chronological order. Imaging session: DW-MRI or choline spectroscopy or ^{18}F -FLT PET imaging experiments.

corrected signals were processed with jMRUI. The signals were imported into jMRUI and pretreated by HLSVD (Hankel Lanczos Singular Value Decomposition) to remove the residual water peak. The HLSVD step was necessary to obtain stable fits of the metabolite peaks. Model fitting was performed using the QUEST routine of jMRUI. This routine works on the time-domain data and adjusts the amplitudes for a set of model metabolite signals. Peak areas were measured for choline peak (at 3.21 ppm). Metabolic ratios were calculated based on measurements of the unsuppressed water peak area.

4.6. ^{18}F -FLT PET Imaging

Each tumor was assessed by ^{18}F -FLT PET imaging to obtain the T/B ratio. Mice were injected intravenously in a tail vein with 7.4–12.9 MBq ^{18}F -FLT that was produced as previously described (36).

PET acquisitions were performed 1 h after tracer injection on a dedicated small-animal PET scanner (Mosaic, Philips Medical System, Cleveland, OH, USA) with a spatial resolution of 2.5 mm (FWHM; 37) full-width at half-maximum. Mice anesthetized with 2% isoflurane underwent a 10 min emission scan followed by a 10 min transmission scan using a 370 MBq ^{137}Cs source for attenuation correction. After corrections of raw data for system dead time, random, scatter coincidences and attenuation, images were reconstructed using a fully 3D iterative algorithm (3D-RAMLA) in a $128 \times 128 \times 120$ matrix, with a voxel of 1 mm^3 . The uptake of tracer in tumors was expressed as T/B ratio, calculated as the mean activity in the tumor region divided by the mean activity in the background region. Regions of interest were delineated using the program PMOD (PMOD Technologies Ltd, Zurich, Switzerland) on consecutive transversal slices, creating a volume of interest (VOI) encircling the tumor. The 2D ROIs were established on consecutive transversal slices using a 50% isocontour tool (ROI including the pixel values greater than 50% of the maximum pixel) that semiautomatically defined a 3D VOI around the tissue of interest. The background VOI was chosen in a muscle region to be representative of the tracer presence within non-tumor tissue.

4.7. Irradiation

The FSall tumor-bearing leg was irradiated locally with 20 Gy of ^{137}Cs irradiator IBL-637 (Oris, France). The dose rate for animal irradiation setting was about 0.6 Gy min^{-1} . The tumor was centered in a 3 cm circular irradiation field. A single-dose irradiation of 20 Gy was given 24 h after the second injection of sorafenib treatment.

4.8. Histology

Tumor-bearing animals were treated with sorafenib during 2 days ($n=4$), remained untreated ($n=4$), were treated with sorafenib during 2 days and irradiated ($n=4$) or remained untreated but irradiated ($n=4$). Tumors were then excised 24 h post-treatment. Tumors were either fixed in 4% paraformaldehyde for standard paraffin sections or embedded in OCT (Optimum cutting temperature) compound for cryosectioning. In both cases, samples were cut into $5 \mu\text{m}$ sections. The paraffin sections were stained with anti-Ki67 antibody (Abcam ab16667) and photographed on a Zeiss MIRAX slide scanner for a global overview of the proliferative regions in the tumors. The frozen slices were probed for apoptosis by TUNEL assay using a commercially available *in situ* cell death detection kit (Roche Diagnostics, Vilvoorde, Belgium).

Nuclei were also counterstained with 4,6-diamidino-2-phenylindole. Slides were photographed using a Zeiss MIRAX slide scanner equipped for fluorescence. Quantification of proliferative and apoptotic regions expressed as percentage of the whole tumor area was obtained using Frida software.

4.9. Tumor Growth Delay

At day 0, corresponding to a mean tumor diameter of $8 \pm 0.5 \text{ mm}$, tumor-bearing mice ($n=8$ per group) were injected with sorafenib (45 mg kg^{-1} per day) for 6 days or with vehicle (DMSO). The diameter of the hind leg was measured daily. When the rear leg diameter reached 15 mm, the mice were killed.

4.10. Statistical Analysis

Results are given as means $\pm$ SE values from n animals. Comparisons between two groups were made with a t -test and with one-way ANOVA (*post-hoc* Dunnett's multiple comparison tests) for multiple groups. p -Values ≤ 0.05 (*), ≤ 0.01 (**) or ≤ 0.001 (***) were considered significant.

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