Hypoxia has emerged as an important factor in tumor biology and in the response to cancer treatment since it has been correlated with angiogenesis, tumor aggressiveness, local recurrence, and metastasis [1–3]. Because of the so-called “oxygen enhancement effect”, the radiation dose required to achieve the same biological effect is about three times higher in the absence of oxygen than in the presence of normal levels of oxygen [4,5]. Hypoxic tumor cells, which are therefore more resistant to radiotherapy than well oxygenated ones, remain clonogenic and contribute to the therapeutic outcome of fractionated radiotherapy [6]. Direct evidence of hypoxia in human cancer has been largely demonstrated in the past [2,7]. A particular area of interest is therefore to combine radiotherapy with co-treatments that are able to transiently increase tumor pO2 at the time of irradiation [8–10].

Farnesyl-transferase inhibitors (FTI), which are described as RAS inhibitors have been shown to be able to reduce tumor hypoxia in experimental models using the hypoxic cell marker pentfluorinated 2-nitroimidazole [11]. FTI have also been shown to potentiate the effect of radiation therapy in independent studies [12]. Activation of RAS leads to the activation of several effector pathways, the best characterized of which being the RAF/MEK/ERK pathway or “classical Mitogen-Activated Protein Kinase” pathway [13]. This pathway, generally expressed in all cell types, transduces extracellular stimulations, e.g. growth factors, cytokines and stress signals, into intracellular responses [14]. MAPK have a central role in integrating extracellular stimulations into various cellular activities, such as gene expression, cell proliferation, survival and migration [15]. Phosphorylation and consecutive activation of ERK regulates the transcription of target genes that promote cell cycle progression and cell survival [13]. MAPK inhibitors have subsequently been developed and are currently used in the clinics in different types of cancers [13–15].

Until now, no study focused on the link between a potential tumor reoxygenation following the administration of MAPK inhibitors and tumor radiosensitization. This was the aim of the current work which was designed to study the effect of MAPK inhibitors in terms of tumor oxygenation and radiation response.

Hypoxia following administration of Mitogen-Activated Protein Kinase inhibitors: A rationale for combination with radiation therapy

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ABSTRACT

Background and purpose: The relevance of Mitogen Activated Protein Kinase (MAPK) inhibitors as co- treatments for radiation therapy is investigated, with special focus on a potential link between the MAPK pathway and tumor hypoxia, which is a critical determinant for response to therapy.

Materials and methods: The effects of two MAPK inhibitors, Sorafenib and PD0325901, were monitored daily using in vivo EPR (Electron Paramagnetic Resonance) oximetry in FSaII and TLT tumor models in order to identify a window of reoxygenation, during which tumor blood flow, oxygen consumption and radiation sensitivity were assessed.

Results: Reoxygenation was shown after two days of treatments with Sorafenib or PD0325901 in two tumor models, which was further successfully exploited with Sorafenib for improving the radiation response of FSaII tumors by a factor of 1.5. The increase in tumor oxygenation was shown to be the result of two major factors: (i) an increase in blood flow for Sorafenib, that might be linked to its anti-angiogenic effect (vascular normalization), and (ii) a decrease in oxygen consumption for Sorafenib and PD0325901, due to an alteration of the mitochondrial activity.

Conclusion: We evidenced tumor reoxygenation in vivo following MAPK inhibition and suggest a rationale for the combination of radiation therapy with Sorafenib.

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For this purpose, we choose 2 different MAPK inhibitors with different specificities, namely Sorafenib which is already used in the clinic, and PD0325901, which is tested in experimental studies.

Sorafenib (Nexavar) was the first RAF kinase inhibitor to enter human clinical testing and is now approved for use in renal cell and hepatocellular carcinoma [13]. This compound, initially developed as a selective inhibitor of RAF, has shown other biologically relevant targets, including vascular endothelial growth factor receptors (VEGFR2/3), platelet-derived growth factor receptor (PDGFR), Flt-3, c-kit, and FGFR-1 [14]. PD0325901 is a specific MEK1/2 inhibitor that blocks the conversion of ERK to its activated, phosphorylated form by inhibiting activated MEK1 and MEK2 [15].

In the current study, we monitored the effect of Sorafenib and PD0325901 on tumor oxygenation, using in vivo Electron Paramagnetic Resonance oximetry [16,17] on two different experimental hypoxic tumor models. After identification of a window of reoxygenation by daily measurements of tumor \( pO_2 \), we assessed the therapeutic relevance of such an effect by measuring the regrowth delays after X-ray irradiation of the tumors during the reoxygenation window induced by Sorafenib, and investigated potential underlying mechanisms responsible for the reoxygenation effect.

Materials and methods

Animal and tumor models

A transplanted liver tumor model (TLT) [18], and a fibrosarcoma (FSaII) [19], were inoculated in the leg of NMRI and C3H/HeOuJco mice, respectively. For inoculation, \( 10^6 \) cells in 0.1 mL of serum free 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 mixed with 21% oxygen (air). All animal experiments were conducted in accordance with national and university animal care regulations.

Treatments

Sorafenib, U0126 (LC Laboratories, MA, USA); and PD0325901 (Selleck, TX, USA) dissolved in DMSO (Invitrogen), were delivered i.p at a dose of 45 mg/kg for Sorafenib and 20 mg/kg for PD0325901 via 100 \( \mu \)L injections. Control mice were treated with 100 \( \mu \)L/day of DMSO.

Western blot analyses

FSaII cells were cultured in DMEM containing 10% FBS, 4.5 mg/L glucose and 1% penicillin–streptomycin and treated during 24 h with U0126 (10 \( \mu \)M) or Sorafenib (10 \( \mu \)M) or PD0325901 (10 nM) in 6 well plates. Appropriate protein amounts (20 \( \mu \)g) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Immunodetection was performed using the ECL detection kit (Amersham, UK), rabbit polyclonal antibodies against ERK and phospho-ERK (Cell Signaling Technology; Danvers, MA, USA), and rabbit secondary antibody (Chemicon, Billerica, MA, USA).

In vivo tumor \( pO_2 \) measurement

In vivo tumor \( pO_2 \) was monitored daily using EPR oximetry. The technique relies on the oxygen-dependent broadening of the EPR line width of a paramagnetic oxygen sensor implanted in the tumor [20]. The technique is intended for continuous measurement of local \( pO_2 \) without altering the local oxygen concentration. EPR spectra were recorded using an EPR spectrometer (Magnetech, Berlin, Germany) and with a low frequency microwave bridge operating at 1.1 GHz and an extended loop resonator. A charcoal (Charcoal wood powder, CX0670-1, EM Science, Gibbstown, NJ) [21] was used as the oxygen-sensitive probe in all experiments. Calibrations curves were made by measuring the EPR line width as a function of the \( pO_2 \). Mice were injected in the center of the tumor (7 mm diameter) using the suspension of charcoal (100 mg/ml, 50 \( \mu \)L injected, 1–25 \( \mu \)l particle size). The tumor under study was placed in the center of the extended loop resonator whose sensitive volume extends 1 cm into the tumor mass. The charcoal was dispersed in a volume of approximately 10 mm\(^3\). The \( pO_2 \) measurements correspond to an average of \( pO_2 \) values in this volume of the tumor. Data acquisition was performed every day before the injection of MAPK inhibitors for each inhibitor or vehicle during 4 days, on both TLT and FSaII tumor models: Sorafenib \( (n=4 \text{ for TLT and } n=5 \text{ for FSaII}) \) and PD0325901 \( (n=6 \text{ for TLT and } n=4 \text{ for FSaII}) \). Control groups received daily DMSO injections \( (n=4 \text{ for TLT and FSaII}) \).

Tumor blood flow estimation

Patent Blue (Sigma–Aldrich, Belgium) staining was used to obtain a rough estimate of FSaII tumor perfusion fraction at day 2 after treatment with Sorafenib \( (n=5) \), PD0325901 \( (n=5) \) or vehicle \( (DMSO, n=14) \), using a technique previously described [22]. Briefly, 1 min after injection of a Patent Blue solution (1.25%) into the tail vein, mice were sacrificed. Tumors were carefully excised and the stained versus unstained tumor areas were quantified on digital pictures of tumor hemi-sections, using an in-house Matlab routine (Mat work, Natick, MA, USA). For each tumor, the percentage of stained area of the whole cross section was determined and was used as an indicator of tumor perfusion.

Ex vivo vessel quantification by Immunohistochemistry

FSaII tumor-bearing mice were sacrificed after 2 days of treatment with Sorafenib \( (n=5) \) or vehicle \( (n=5) \). Dissected tumors embedded in Tissue-Tek OCT compound were frozen in liquid nitrogen-cooled isopentane. After fixation in acetone, cryosections (5 \( \mu \)m) were incubated overnight at 4 \( ^\circ \)C with a rat anti-CD105 monoclonal antibody (clone M7/18, Hybridoma, Iowa, 7 \( \mu \)g/ml) followed by 45 min incubation at room temperature with Alexa-568 conjugated anti-rat immunoglobulin (Invitrogen, 1/300). Antibodies were diluted in PBST/BSA1%. Nuclei were stained with Hoechst 33342. Slides were mounted using Vectashield mounting medium and scanned with a Zeiss Mirax fluorescence microscope. Quantification of vessels was obtained by calculating the surface of red labeling (CD105) per tumor slice using the Frida software.

Ex vivo oxygen consumption rate evaluation

The method described by Jordan et al. [23] was used. All spectra were recorded on a 9 GHz Bruker EMX EPR spectrometer. FSaII tumor-bearing mice were treated for 2 days with MAPK inhibitors \( (n=4 \text{ for Sorafenib and } n=3 \text{ for PD0325901 and vehicle}) \). At day 2 after treatment, the mice were sacrificed and the tumor was excised, trypsinized for 20 min, from which a 1.10\(^7\) cells/ml suspension was obtained for control and treated tumors, cell viability was determined with Trypan blue staining. Then cells were suspended in 10% dextran in complete medium [23,24]. We also used an in vitro specific and powerful inhibitor of MEK 1/2 called U0126 in order to show the direct link between MAPK inhibition and oxygen consumption inhibition. A neutral nitroxide (oxygen reporter probe), \(^{15}\)N 4-oxo-2,2,6,6-tetramethylpiperidine-\( d_{15}\)-\(^{15}\)N-1-oxyl at 0.2 mmol/L (CDN Isotopes, Pointe-Claire, Quebec, Canada), was added to 100 \( \mu \)L aliquots of tumor cells that were then drawn into glass capillary tubes. The probe in complete medium (0.2 mM in 10% dextran to avoid settling of the cells in the EPR cavity) was calibrated at
various O₂ between 100% nitrogen and air so that the line width measurements could be related to O₂ at any value. Nitrogen and air were mixed in an Aalborg gas mixer, and the oxygen content was analyzed using a servomex oxygen analyzer OA540. The sealed tubes were placed into quartz EPR tubes, and samples were maintained at 37 °C. As the resulting line width reports on O₂ concentration (as described before [23]), oxygen consumption rates were obtained by measuring the O₂ concentration in the closed tube over time and determining the slope of the resulting linear plot.

In vivo regrowth delay assay

The FSaII tumor-bearing leg was irradiated locally with 20 Gy of 250 kV X-rays (RT 250; Philips Medical Systems, Germany). 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. After radiotherapy, tumor growth was determined by daily measurement of tumor diameters. A linear fit was estimated between 8 and 15 mm (at which time the mice were sacrificed), which allowed determination of the time to reach a particular size (i.e. regrowth delay to reach 12 mm in diameter) for each tumor.

In vitro intracellular glutathione measurement

The glutathione content was determined using the Tietze enzyme recycling assay [25], with slight modifications [26]. Cells were washed twice with ice-cold phosphate buffered saline and then lysed with a solution of 5-sulfosalicylic acid (5%). After two freeze-thaw cycles, samples were centrifuged at 10 000 x g for 10 min and the resulting supernatants were kept at -80 °C until used. Ten microliters of the samples were then placed in a reaction mixture containing 0.2 U/ml of glutathione reductase, 50 μg/ml 5,5’-Dithio-bis(2-nitrobenzoic acid) and 1 mM EDTA at pH 7. The reaction was initiated by the addition of 50 μM NADPH and changes in absorbance were recorded at 412 nm. Results were normalized to the protein content using the method of Lowry [27].

Ex vivo apoptosis measurement

FSaII tumor-bearing mice were treated with Sorafenib or vehicle (n = 4/group) and tumors were excised at day 2 after treatment initiation. Tumors were embedded in Tissue-Tek OCT compound and frozen in liquid nitrogen-cooled isopentane for cryosectioning. Samples were cut into 5 μm sections. The frozen slices were probed for cellular death by TUNEL assay using an in situ cell death detection kit (Roche Diagnostics, Belgium). Nuclei were also counterstained with Hoechst 33342. Slides were then scanned with a Zeiss Mirax fluorescence microscope. Quantification of apoptosis was obtained by calculating the surface of red labeling (TUNEL) per tumor slice using FRIDA software.

Statistical analysis

Results are given as means ± SE values from n animals. Comparisons between groups were made with one-way ANOVA along with post-hoc Dunnett’s multiple comparison tests or two-way ANOVA where appropriate. P values <0.05 (*), <0.01 (**) or <0.001 (***) were considered significant.

Results

Western blot analysis

We observed an inhibition of ERK phosphorylation (Fig 1A and B) in FSaII tumor cells, after treatment with the RAF inhibitor, Sorafenib (10 μM), the MEK inhibitor, PD0325901 (10 nM), and the in vitro MEK inhibitor (positive control), U0126 (10 μM).

Effect of MAPK inhibitors on tumor oxygenation

After daily administration, we observed an increase in pO₂ in TLT and FSaII tumors for both inhibitors (Fig 2C) from day 1 until day 3 post beginning of treatment (this increase was not observed for the control group). The pO₂ after two days of treatments was significantly higher than before treatment (p < 0.05, Fig 2A and B). We chose day 2 as the “window of reoxygenation” for the rest of the experiments because there was no further significant increase between day 2 and 3.

Using Hypoxyprobe™-1 Omni Kit, we also observed a net decrease in tumor hypoxia as shown by the distribution of the probe in non-necrotic areas of tumor biopsies after two days of treatment with Sorafenib (51 supplementary material).

Since an increase in tumor oxygenation could be due to an increase in oxygen supply (blood flow) and/or to a decrease in oxygen consumption by tumor cells, both parameters were assessed in the following experiments for both inhibitors.

Effects of MAPK inhibitors on FSaII tumor perfusion

An estimation of tumor perfusion fraction was carried out using the Patent Blue staining assay, a method that was previously compared with DCE (Dynamic Contrast Enhanced) MRI data [28,29]. Of note, the time between dye injection and mouse sacrifice being really short (1 min), this technique is described to be more sensitive to perfusion fraction than to permeability factors. Tumors treated with Sorafenib stained more positive (n = 5; colored area: 73 ± 15.5%) than tumors treated with vehicle (n = 14; colored
area: 11.2 ± 0.9%, (p < 0.01). By contrast, we did not observe any significant modification in perfusion fraction after treatment by PD0325901 (Fig 3A).

Effect of Sorafenib on FSaII tumor vascular density

Because of the increase in tumor perfusion in the Sorafenib group, we measured vascular density after treatment with Sorafenib using immunohistochemistry with CD105 antibody and showed a vascular density of 8% (Vascular surface area/tumor) in control tumors and of 2.4% in treated tumors (p < 0.0001) (Fig 3B).

Effect of MAPK inhibitors on tumor cell oxygen consumption

The in vivo administration of drugs significantly decreased the ex vivo oxygen consumption rate of FSaII cells, with a slope of 0.65 μM/min for the control group, 0.10 μM/min for Sorafenib (p < 0.01) and 0.10 μM/min for PD0325901 (p < 0.01) (Fig 4A–C). Another MEK inhibitor, U0126 (positive control), used in vitro at 10 μM after 6 h also decreased the oxygen consumption rate: 0.98 μM/min for the control group and 0.49 μM/min for U0126 (data not shown).

A decrease in mitochondrial activity, assessed by measuring the intensity of a fluorescence probe (Mitotracker™ Orange) was observed in FSaII cells treated with Sorafenib at 10 μM during 2 h, compared with the intensity of fluorescence in cells treated with vehicle: (DMSO) (S2 supplementary material).

Tumor regrowth delay assay after treatment with Sorafenib

In order to assess the therapeutic relevance of Sorafenib as a potential co-treatment for radiation therapy during the window of reoxygenation, we performed a regrowth delay (rd) assay in FSaII
For that purpose, we combined Sorafenib with 20 Gy of X-rays irradiation (at day 2). Tumor growth curves are presented on Fig 5A. Without irradiation, 2 days of treatment with Sorafenib did not significantly affect tumor growth, as the times to reach 12 mm in tumor diameter were 5.6 ± 0.2 days (n = 5) and 6.3 ± 0.5 days (n = 6) for the treated and control groups, respectively. When irradiated with 20 Gy of X-rays (after 2 days of vehicle injection), FSaII tumor growth was significantly delayed since the time to reach 12 mm was 15.7 ± 1.6 days (n = 8). Pre-treatment with Sorafenib during 2 days before irradiation led to a significant further increase (p < 0.01) in tumor rd with a time to reach 12 mm in diameter of 23.4 ± 1.8 days (n = 9). The rd was enhanced by a factor of 1.5 in the group that had been pre-treated with Sorafenib during 2 days and irradiated compared to the control (vehicle treated) irradiated group. In order to prove that the radiosensitizing effect of Sorafenib was due to an oxygen effect, we included a group (pre-treated with Sorafenib and irradiated) that was clamped at the time of irradiation in order to deprive the tumor of oxygen. We did not observe any enhancement in rd compared to the control (vehicle treated) irradiated group, with a rd of 11 ± 1.3 days (n = 4) for this group. The median survival time (time for the tumor to reach 15 mm) provided by the Kaplan–Meier curve (Fig 5B) was about 20.5 days for the irradiated control group and 29.5 days for the Sorafenib pre-treated and irradiated group. As additional control, we also compared in an independent experiment, two groups of irradiated mice: untreated tumors (X-Rays: 11.6 days ± 0.9) with untreated clamped tumors (X-Rays+Ligation: 9.5 days ± 0.8), in order to observe the effect of the ligation of untreated tumors. We did not observe any significant modification of rd between these groups (data not shown).

Effect of Sorafenib on FSaII intracellular GSH content

In order to determine whether the decrease in oxygen consumption by tumor cells after treatment with Sorafenib could partly be explained by a reduction in intracellular GSH content, we measured the intracellular concentration of GSH after treatment with tumors. For that purpose, we combined Sorafenib with 20 Gy of X-rays irradiation (at day 2). Tumor growth curves are presented on Fig 5A. Without irradiation, 2 days of treatment with Sorafenib did not significantly affect tumor growth, as the times to reach 12 mm in tumor diameter were 5.6 ± 0.2 days (n = 5) and 6.3 ± 0.5 days (n = 6) for the treated and control groups, respectively. When irradiated with 20 Gy of X-rays (after 2 days of vehicle injection), FSaII tumor growth was significantly delayed since the time to reach 12 mm was 15.7 ± 1.6 days (n = 8). Pre-treatment with Sorafenib during 2 days before irradiation led to a significant further increase (p < 0.01) in tumor rd with a time to reach 12 mm in diameter of 23.4 ± 1.8 days (n = 9). The rd was enhanced by a factor of 1.5 in the group that had been pre-treated with Sorafenib during 2 days and irradiated compared to the control (vehicle treated) irradiated group. In order to prove that the radiosensitizing effect of Sorafenib was due to an oxygen effect, we included a group (pre-treated with Sorafenib and irradiated) that was clamped at the time of irradiation in order to deprive the tumor of oxygen. We did not observe any enhancement in rd compared to the control (vehicle treated) irradiated group, with a rd of 11 ± 1.3 days (n = 4) for this group. The median survival time (time for the tumor to reach 15 mm) provided by the Kaplan–Meier curve (Fig 5B) was about 20.5 days for the irradiated control group and 29.5 days for the Sorafenib pre-treated and irradiated group. As additional control, we also compared in an independent experiment, two groups of irradiated mice: untreated tumors (X-Rays: 11.6 days ± 0.9) with untreated clamped tumors (X-Rays+Ligation: 9.5 days ± 0.8), in order to observe the effect of the ligation of untreated tumors. We did not observe any significant modification of rd between these groups (data not shown).

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Effect of Sorafenib on FSaII intracellular GSH content

In order to determine whether the decrease in oxygen consumption by tumor cells after treatment with Sorafenib could partly be explained by a reduction in intracellular GSH content, we measured the intracellular concentration of GSH after treatment with
Sorafenib at 5 and 10 μM during 24 h (n = 3). GSH contents were 0.91 and 0.70 mmol/mg proteins for Sorafenib at 5 and 10 μM, respectively, and 1.66 mmol/mg proteins for control cells. Cells treated with BSO (positive control) were measured at 0.12 mmol GSH/mg protein (Fig 6A).

**Effect of Sorafenib on apoptosis cell death (TUNEL assay)**

Using a TUNEL assay, we observed a small increase in apoptosis cell death in tumors treated during two days with Sorafenib (0.96%) compared with control tumors (0.66%), p < 0.05 (Fig 6B).

**Discussion**

The present study is the first to demonstrate the ability of MAPK inhibitors to improve tumor oxygenation in vivo in experimental models. Indeed, we showed an increase in pO2 value for Sorafenib and PD0325901 in two experimental tumor models using EPR oximetry, which was illustrated for Sorafenib by immunohistochemical staining images with pinoponidazole. The window of reoxygenation at day 2 was further successfully exploited with Sorafenib for improving the radiation response of FSaII tumors. Indeed, we observed an enhancement in regrowth delay after a 20 Gy irradiation of tumors from the Sorafenib treated group (2 days of treatment) compared to the control group (sham treated during 2 days with DMSO). Furthermore, an additional group of mice treated with Sorafenib, irradiated at day 2 and for which the tumors were clamped at the time of irradiation (in order to transiently deprive them with oxygen), did not show any increase in regrowth delay compared to the control group (sham treated and irradiated). We thereby proved that the decrease in oxygen delivery to the tumor during irradiation in treated tumors was able to completely reverse the radiosensitizing effect of the drug, suggesting a major oxygen effect mechanism at the origin of the radiosensitizing properties of Sorafenib.

Previous studies showed that Sorafenib combined with fractional radiotherapy was able to enhance the regrowth delay of treated tumors [30,31], an effect attributed to the anti-angiogenic properties of the drug because of a lack of demonstration of any radiosensitizing properties of Sorafenib in vitro even though a significant increase in the regrowth delay was observed in vivo [24]. Other studies had previously related the effect of anti-angiogenic drugs on tumor oxygenation and radiation response [32–36].

In our study, the increase in tumor oxygenation was shown to be the result of two major factors: an increase in tumor blood flow for Sorafenib only, concomitant to a decrease in oxygen consumption for Sorafenib as well as for PD0325901. As inhibitors of oxygen consumption block electron transfer in mitochondria [37], the mitochondrial activity was assessed using the Mitotracker probe in FSaII tumor cells treated with Sorafenib, and a significant decrease was observed. The mitochondrial effect could in turn be due to the observed glutathione (GSH) depletion in FSaII Sorafenib treated cells. Indeed, the generation of ROS usually oxidizes GSH to GSSG and ultimately reduces the total GSH level. GSH depletion has been shown to directly modulate the loss of the mitochondrial membrane potential [38]. Sorafenib has been shown to be implicated in a mitochondria-dependent oxidative stress mechanism in HepG2 cells (hepatocellular carcinoma), which is caused by a rapid production of ROS followed by a nearly 90% GSH depletion [39]. The concentrations tested were determined in accordance with the plasma level of Sorafenib in patients which received a standard dose of Sorafenib (400 mg twice daily) [40]. Similarly, we observed a significant decrease in glutathione rate in FSaII tumor cells treated with Sorafenib during 24 h, which reduces cells defences against oxidative stress and could alter the mitochondrial membrane potential [44] that is crucial for mitochondrial respiratory function. In addition, Sorafenib has been shown to down regulate mcl-1 protein expression in numerous cell lines [41–45], whose expression is essential for the integrity of the mitochondrial membrane potential. All these data suggest a role of the mitochondrial dysfunction as a potential factor at the origin of the decrease in oxygen consumption by tumor cells consecutive to treatment with MAPK inhibitors.

A second factor able to influence pO2 is the modification of blood flow that regulates oxygen delivery to tumor. We did not observe any modification in blood flow for tumors treated by PD0325901. However, Sorafenib was able to significantly increase tumor blood flow after two days of treatment. The antiangiogenic effect of Sorafenib which is also a VEGFR and PDGFR inhibitor is likely to be involved in blood flow enhancement. Accordingly, anti-VEGF therapy has been shown to improve the localization of drugs for which abnormal vasculature is a barrier to delivery and, by increasing tissue oxygenation, to enhance the response to radiotherapy [46]. This is in accordance with the ‘normalization’ theory developed by R. Jain suggesting an early normalization phase of the tumor vessels [47]. Drugs that induce vascular normalization can alleviate hypoxia and increase the efficacy of conventional therapies if both are carefully scheduled [35,36,47]. In the current study, in order to see whether the increase in tumor blood flow for the Sorafenib treated tumors could be due to a normalization effect, we measured the vascular density after treatment with Sorafenib using immunohistochemistry. Tumor blood flow enhancement and tumor vascular density decrease, as well as a decrease in the number of small vessels, measured after 2 days.
of daily injection with Sorafenib, suggested a vascular normalization effect of this antiangiogenic drug, although more accurate methods for blood flow measurement and pericyte analysis would be required to corroborate this hypothesis. However we did not consider the increase in blood flow induced by Sorafenib as the single factor responsible for the radiation response enhancement, but also considered the decrease in oxygen consumption by tumor cells. Indeed PD0325901 was also able to increase tumor oxygenation to the same extent as Sorafenib, without any effect on tumor blood flow, but due to the single targeting of oxygen consumption. We observed a significant increase in cell death (assessed by TUNEL assay) in tumors after a two-day treatment with Sorafenib. Nevertheless, the apoptosis rate below 1% for both control and treated group and this difference is not likely to influence oxygen consumption. Furthermore cells were counted to have the same number for control and treated tumors just before measurement of oxygen consumption.

To conclude, we identified a window of reoxygenation after 2 days of treatment with Sorafenib and PD0325901, two MAPK inhibitors. This effect was shown to be partly due to a major decrease in oxygen consumption by tumor cells. The successful exploitation of the reoxygenation window induced by Sorafenib to radiosensitize tumors suggests the potential relevance of using Sorafenib as a co-treatment for radiation therapy, after careful identification of the timing of reoxygenation. This effect might be extended to other MAPK inhibitors since similar reoxygenation properties were observed after administration of PD0325901. If further experiments would be required to corroborate a class effect, this study provides important insights for the sequence of administration of the treatments and underlines the importance of individual monitoring of tumor oxygenation while combining treatments with radiation therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2012.05.005.

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