

SHORT REPORT

Use of Xanthinol Nicotinate as a co-treatment for radio- and chemo-therapy in experimental tumors

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The tumor micro-environment plays a key role in the tumor resistance to cytotoxic treatments. It has been demonstrated that it is possible to modulate the tumor microenvironment to potentiate anti-cancer therapy. Here, we made the hypothesis that the vasoactive agent xanthinol nicotinate (XN) could be an important modulator of the tumor perfusion and oxygenation. Using functional non invasive techniques (*in vivo* EPR oximetry and dynamic contrast enhanced MRI), we were able to define a time window in which tumor oxygenation, flow and permeability were significantly increased in the TLT tumor model implanted in muscles of mice. As a consequence of the alleviation of tumor hypoxia, we found out that XN was able to radiosensitize the tumors when applying 10 Gy of X-Rays during the reoxygenation of the tumors (enhancement in radiation response of 1.4). Moreover, the administration of cyclophosphamide (50 mg/kg) used as a chemotherapeutic agent was more efficient when applying the treatment after XN administration (enhancement in response to chemotherapy of 2.7). These results show the importance of the dynamic evolution of the tumor microenvironment on the response to treatments, and that XN is an efficient modulator of the tumor hemodynamics that may potentiate cytotoxic treatments.

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It is now well established that regions within solid tumors experience mild to severe oxygen deprivation owing to aberrant vascular function. The hypoxic regions are associated with altered metabolism, as well as increased resistance to radiation and chemotherapy.^{1–3}

New strategies for transiently opening the tumor vascular bed to alleviate tumor hypoxia, which is a source of resistance to radiotherapy via the ‘oxygen effect’, and improve the delivery of chemotherapeutic agents, are now considered in the pre-clinical setting. This so-called ‘pro-vascular’ approach has been named by opposition to the well known ‘anti-vascular’ and ‘anti-angiogenic’ strategies, using agents that are directed at the pre-existing tumor vasculature and at the process of vascular development, respectively,⁴ although recent insights into the normalization of the tumor vasculature suggest the early effects of anti-angiogenic agents and pro-vascular agents might be similar.^{5–7} In the last years, our group has identified several pro-vascular agents that are able to improve the issue of radio and/or chemotherapy in experimental tumors, including NO-mediated agents, modulators of the thyroid status, and anti-angiogenic agents in their normalization phase, among others.^{6,8–13}

In the current study, we hypothesized that xanthinol nicotinate (XN), a vasodilator used in the management of peripheral and cerebral vascular disorders, could improve tumor perfusion and oxygenation in order to get an improved radiotherapy and chemotherapy outcome. XN is the most potent form of niacin, the active form of vitamin B3, and acts as nicotinic acid. XN increases the cerebral metabolism of glucose, ATP levels and cerebral blood flow.^{14,15}

We assessed the effect of XN on radiotherapy and cyclophosphamide chemotherapy in the TLT mouse tumor model. The first

step was to monitor tumor pO₂ after administration of XN by electron paramagnetic resonance (EPR) oximetry to determine the window of the vascular bed opening. As demonstrated by Patent Blue staining and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI), the observed increase in pO₂ was the signature of an increased tumor blood flow. These hemodynamic changes were then exploited in order to potentiate radiotherapy and chemotherapy, as demonstrated by the determination of regrowth delays.

Material and methods

Mice and tumors

Syngeneic TLT (Transplantable mouse Liver Tumor),¹⁶ were injected intramuscularly in the thigh of 5 week-old male NMRI mice (Animalerie facultaire, Université catholique de Louvain, Brussels). Tumor diameter was measured daily with a digital caliper. All animal experiments were performed in accordance with national animal care regulations.

XN and cyclophosphamide injections

XN was dissolved in saline and injected intraperitoneally at a final concentration of 75 mg/kg.¹³ The alkylating agent cyclophosphamide was dissolved in saline and injected intraperitoneally at the suboptimal dose of 50 mg/kg.⁷

Tumor oxygenation

Electronic Paramagnetic Resonance (EPR) Oximetry, using charcoal (CX0670-1, EM Science, Gibbstown, NJ) as the oxygen sensitive probe, was used to evaluate the tumor oxygenation.¹⁷ EPR oximetry relies on the oxygen-dependent broadening of the EPR line width of a paramagnetic oxygen sensor implanted in the tumor. This technique is designed for continuous measurement of the local pO₂ without altering the local oxygen concentration, and allows repeated measurements from the same site over long periods of time. EPR spectra were recorded using an EPR spectrometer (Magnetech, Berlin, Germany) with a low frequency microwave bridge operating at 1.2 GHz and extended loop resonator. Mice were injected once in the center of the tumor 1 day before measurement using the suspension of charcoal (suspension in saline containing 3% Arabic gum, 100 mg/ml, 50 µl injected, 1–25 µm particle size). The localized EPR measurements correspond to an average of pO₂ values in a volume of ~10 mm³.¹⁷ Data acquisition was performed every 5 five minutes for 1 hour after injection of XN (n = 5) or vehicle (n = 5). Tumor bearing mice were anesthetized using isoflurane (Forène, Abott,

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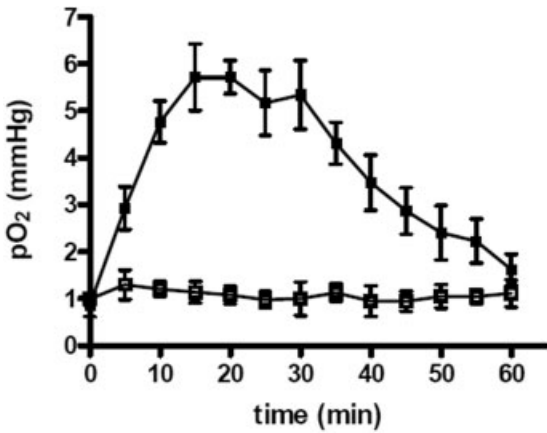


FIGURE 1 – Evolution of pO₂ in tumors treated with XN (■) and vehicle (□) over time, monitored by EPR oximetry.

Louvain-La-Neuve, Belgium) delivered using a calibrated vaporizer at 3.0% in air for induction and 1.5% for maintenance.

Patent blue staining

Patent Blue (Sigma-Aldrich, Belgium) was used to obtain a rough estimate of the tumor perfusion¹⁸ after 2 days of treatment with thalidomide or vehicle. This technique involves the injection of 200 μ L of Patent Blue (1.25%) solution into the tail vein of the mice. After 1 minute, a uniform distribution of the staining through the body was obtained and mice were sacrificed. Tumors were carefully excised and cut into size-matched halves. Pictures of each tumor cross section were taken with a digital camera. To compare the stained *versus* unstained area, an in-house program running on Interactive Data Language (Research Systems Inc., Boulder, CO) was developed. For each tumor, a region of interest (stained area) was defined on the two pictures and the percentage of stained area of the whole cross section was determined. The mean of the percentages of the two pictures was then calculated and was used as an indicator of tumor perfusion.⁶

DCE-MRI

The perfusion was monitored in the time window during which intratumoral pO₂ was maximal, as determined by EPR oximetry after administration of XN and in the same time window after administration of vehicle. The perfusion was monitored via single-slice dynamic contrast-enhanced MRI at 4.7 T using the rapid-clearance blood pool agent P792 (Vistarem[®], Guerbet, Roissy, France).¹⁹ High resolution multi-slice T₂-weighted spin echo anatomic imaging was performed just before dynamic contrast-enhanced imaging. Pixel-by-pixel values for K^{trans} (influx volume transfer constant, from plasma into the interstitial space, units of min⁻¹), V_p (blood plasma volume per unit volume of tissue, unitless), and K_{ep} (fractional rate of efflux from the interstitial space back to blood, units of min⁻¹) in tumor were calculated via tracer kinetic modeling of the dynamic contrast-enhanced data,²⁰ and the resulting parametric maps for K^{trans}, V_p, and K_{ep} were generated. Statistical significance for V_p or K^{trans} identified “perfused” pixels, *i.e.*, pixels to which the contrast agent P792 had access.^{6,19}

Irradiation and tumor regrowth delay assay

Irradiation was performed in the time window during which intratumoral pO₂ was maximal (*i.e.*, 20 min after XN administration), as determined by EPR oximetry after administration of XN (group 1, n = 6) and in the same time window after administration of vehicle (group 2, n = 5). Non irradiated groups were also included (XN group, n = 5; and control group, n = 5).

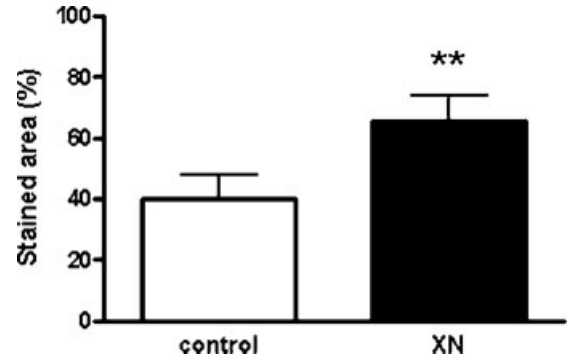


FIGURE 2 – Effect of XN treatment on tumor perfusion assessed by Patent Blue staining. Each bar represents the mean value of tumor percentage of colored area \pm SEM for control group and XN group.

The TLT tumor-bearing leg was locally irradiated with 10 Gy of 250 kV X-rays (RT 250; Philips Medical Systems). The tumor was centered in a 3 cm diameter circular irradiation field. A single dose irradiation was performed. After treatment, the tumor growth was determined daily by measuring tumor diameter until they reached a size of 16 mm, at which time the mice were sacrificed. A linear fit was performed between initial tumor size (7.5 ± 1 mm) and 16 mm which allowed determination of the time to reach a particular size for each mouse.

Chemotherapy and tumor regrowth delay assay

XN treated mice received a single dose of the alkylating agent cyclophosphamide (group 5, n = 5) in the time window during which pO₂ and flow were maximal (*i.e.*, 20 min after XN administration). Vehicle treated mice received the dose of alkylating agent during the same time window (group 6, n = 5). Control experiments consisted in the injection of vehicle (group 7, n = 5) and XN (group 3, n = 5). After treatment, the tumor growth was determined daily by measuring tumor diameter until they reached a size of 16 mm, at which time the mice were sacrificed. A linear fit was performed between initial tumor size (7.5 ± 1 mm) and 16 mm which allowed determination of the time to reach a particular size for each mouse.

Statistical analysis

Results are given as means \pm SEM values from n animals. Comparisons between groups were made with two-way ANOVA and a P value less than 0.05 was considered significant.

Results

Effect of Xanthinol nicotinate on tumor pO₂

Intraperitoneal injection of XN rapidly and transiently modified tumor pO₂, as shown in Figure 1. Maximal pO₂ values were obtained 10 to 30 minutes after XN administration. These data determined the time window of vascular bed opening, which was used to design the “flow” experiments as well as a rationale for scheduling the use of XN as a co-treatment in irradiation and cyclophosphamide chemotherapy experiments.

Effect of Xanthinol nicotinate on tumor hemodynamics

Patent Blue staining. A rough estimate of the tumor perfusion was carried out using the colored area observed in tumors after injection of a dye. Tumors with Xanthinol Nicotinate treatment stained more positive (n = 6; $65.5 \pm 8.6\%$) than tumors treated with vehicle (n = 7; $39.9 \pm 7.9\%$; Fig. 2). This difference was found to be statistically significant ($p < 0.05$).

DCE-MRI. Tumor perfusion was monitored in the TLT tumor model 20 minutes after administration of XN via dynamic contrast-enhanced MRI at 4.7 T using *i.v.* injection of the rapid-

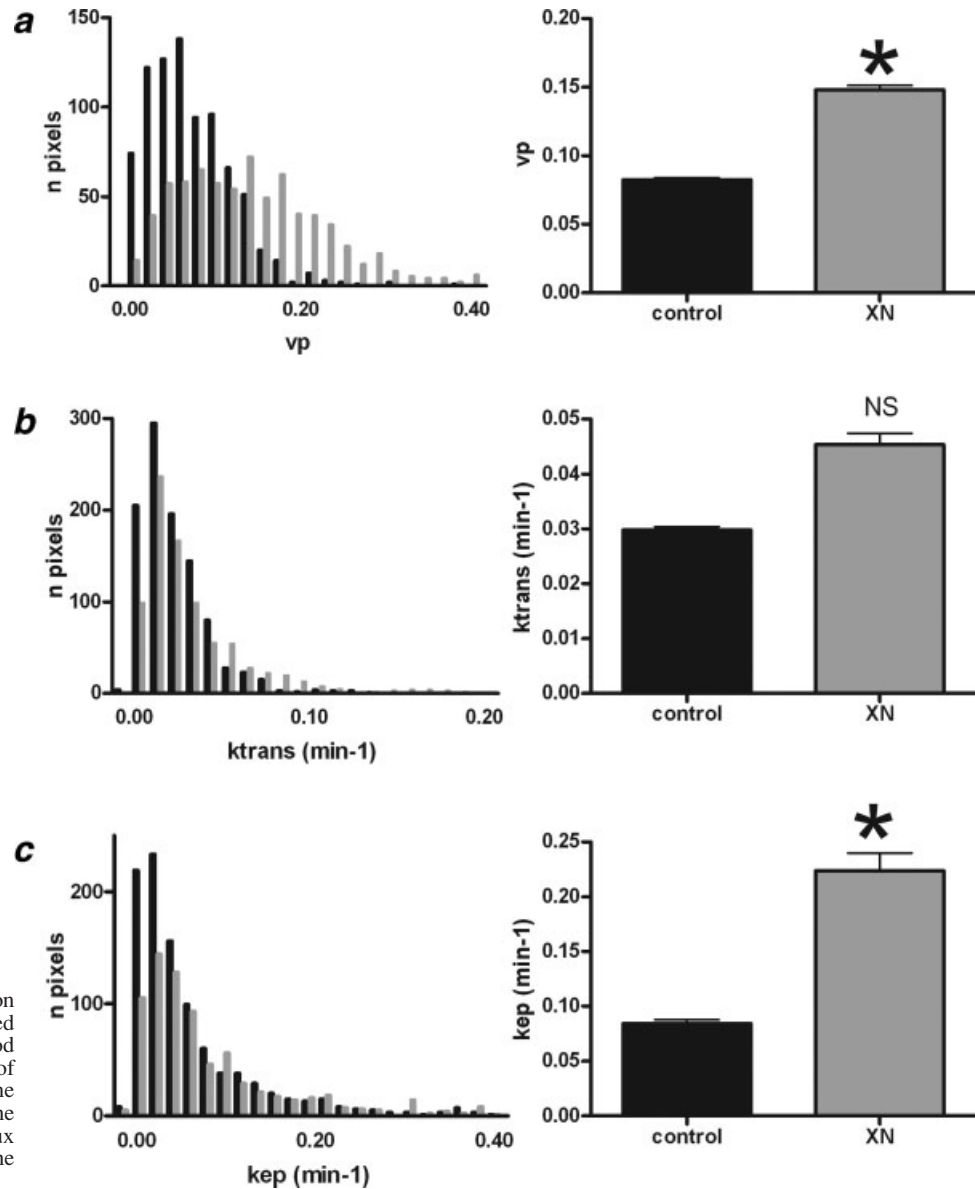


FIGURE 3 – DCE-MRI. Distribution of vascular variables in tumors treated with XN or vehicle. Vp is the blood plasma volume per unit volume of tissue, Ktrans is the influx volume transfer constant from plasma into the interstitial space, and Kep is the efflux volume transfer constant from the interstitial space back to the plasma.

clearance blood pool agent P792 (Vistarem). The pixel-by-pixel analysis generated “perfusion maps” and histograms (using the values for Vp, the blood plasma volume per unit volume of tissue) and “permeability maps” and histograms (using the values for Ktrans, the influx volume transfer constant, from plasma into the interstitial space, and Kep, the efflux volume transfer constant from the interstitial space back to plasma). Significant increases in plasmatic volume fraction (Vp) and kep were observed, showing that both flow and permeability parameters were enhanced (Fig. 3). Post-treatment distribution of Vp is broader and more heterogeneous, showing that all tumor regions do not respond to the same extent. However, heterogeneities in the distributions of pharmacokinetic parameters have previously been shown in experimental as well as in human tumors.

Effect of XN on radiation sensitivity

To assess the therapeutic relevance of the transient increase in tumor oxygenation after XN administration, we combined XN with 10 Gy radiotherapy. Irradiation was performed 20 min after injection of XN or vehicle. Control experiments included injection of XN alone (without irradiation) and injection of saline alone.

The tumor size was monitored every day for all mice. The time for each tumor to reach 14 mm was calculated. The time differences between treated groups and the control group were calculated (regrowth delays or TGD) (Fig. 4). The results show that tumor growth was significantly delayed by XN: TGD were 5.8 ± 1.0 days and 4.2 ± 0.8 days for XN and control groups, respectively; resulting in a factor of enhancement in radiation response of 1.4.

Effect of XN on chemotherapeutic treatment

To evaluate the possible adjuvant effect of XN on chemotherapy, we carried out a protocol that used a suboptimal dose of cyclophosphamide to facilitate the identification of a possible potentiation of combined treatments. This protocol has already been used to show the benefits of drugs that transiently open the tumor vascular bed.^{21,7} Administration of cyclophosphamide was performed 20 min after injection of XN or vehicle. Control experiments were performed by injecting XN or vehicle alone. The tumor size was measured every day for all mice. The time for each tumor to reach 14 mm was calculated. The time differences between treated groups and the control group were calculated (regrowth delays or TGD) (Fig. 5). These results show that

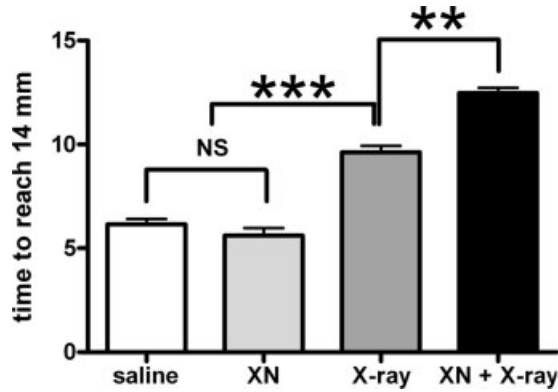


FIGURE 4 – Regrowth delay assay after irradiation experiments. Bar graph of delays (unit in days) needed to reach 14mm tumor diameter after treatment with XN combined or not to 10 Gy of X-rays and their respective controls.

XN potentiates the effect of chemotherapy, as tumors co-treated with XN show a TGD of 4.5 ± 1.6 days vs. 1.7 ± 1 days for cyclophosphamide alone, resulting in a factor of enhancement in response to chemotherapy of 2.7.

Discussion

The use of co-treatments is more and more considered in order to improve the therapeutic efficacy of chemotherapy or radiotherapy. Nevertheless, to be really efficient, a combination of treatments has to be carefully scheduled, *i.e.*, radiotherapy and chemotherapy have to be applied at the exact moment when the vascular bed is opened by the co-treatment. This time window can only be identified by the study of key tumor micro-environment parameters, such as oxygenation and perfusion. In this study, we considered the relevance of combining a peripheric vasodilator, Xanthinol Nicotinate, with radio- and chemo-therapy.

We observed a transient increase in tumor pO_2 from 10 to 30 minutes after administration of Xanthinol Nicotinate, which was correlated with an increased tumor perfusion. The application of radio or chemotherapy in the time window of the vascular bed opening (*i.e.*, 20 minutes after XN administration) resulted in a significant improvement in radiation sensitivity and response to chemotherapy, by a factor of 1.4 and 2.7, respectively.

The selective aspect of the pro-vascular approach rely on the fact that tumor tissues are more hypoxic and less perfused than

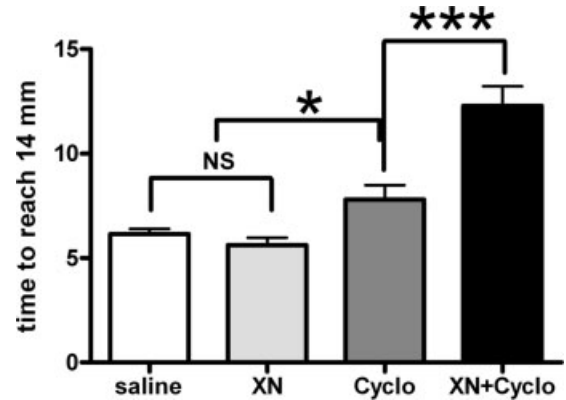


FIGURE 5 – Regrowth delay assay after chemotherapy experiments. Bar graph of delays (unit in days) needed to reach 14mm tumor diameter after treatment with XN combined or not to sub optimal dose of cyclophosphamide and their respective controls.

normal tissues, therefore, the relative enhancement in oxygen and blood flow (respectively related to radiation and chemotherapeutic responses) is larger in tumors than in healthy tissues. Moreover, the critical cut off value in terms of pO_2 and radiosensitization is situated between 5–10 mmHg. Therefore, the impact of a pro-vascular agent able to increase tumor pO_2 above this cut off is substantial compared to normal tissues.

XN has thrombolytic and hypotensive activities in rats and anti-platelet and fibrinolytic effects in patients with peripheral arterial obliterative disease. It has been hypothesized that the mechanism of XN partly consists of a simultaneous release of endogenous prostacyclin and nitric oxide by the nicotinate component of the drug.¹⁴ Our group already characterized several NO-mediated modulators of tumor hemodynamics and radiation response with success, including NO donors and insulin.^{8–10} Although further experiments would be required to fully understand the mechanism of action of the drug within a tumor, the proof of concept of the relevance of using this peripheric vasodilator as a co-treatment to radio and chemotherapy is established in experimental tumors. Moreover, the translation to humans would be direct since the drug is already used safely for several human pathologies.

Nevertheless, additional pre-clinical studies on more elaborated tumor models as well as combination to fractionated radiotherapy or other classes of anti-cancer agents will be required to further characterize the potential use of XN as a potential co-treatment in the clinic.

References

- Menon C, Fraker DL. Tumor oxygenation status as a prognostic marker. *Cancer Lett.* 2005;221:225–35.
- Zhou J, Schmid T, Schnitzer S, Brüne B. Tumor hypoxia and cancer progression. *Cancer Lett.* 2006;237:10–21.
- Bertout JA, Patel SA, Simon MC. The impact of O_2 availability on human cancer. *Nat Rev Cancer* 2008;8:967–75.
- Feron O. Targeting the tumor vascular compartment to improve conventional cancer therapy. *TRENDS Pharmacol.Sci.* 2004;25:536–542.
- Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307:58–62.
- Ansiaux R, Baudalet C, Jordan BF, Beghein N, Sonveaux P, De Wever J, Martinive P, Grégoire V, Feron O, Gallez B. Thalidomide radiosensitizes tumors through early changes in the tumor microenvironment. *Clin. Cancer. Res.* 2005;11:743–50.
- Segers J, Di Fazio V, Reginald A, Martinive P, Feron O, Wallemacq P, Gallez B. Potentiation of cyclophosphamide chemotherapy using the antiangiogenic drug thalidomide: importance of optimal scheduling to exploit the normalization window of the tumor vasculature. *Cancer Lett.* 2006;244:129–35.
- Jordan BF, Grégoire V, Demeure RJ, Sonveaux P, Feron O, O'Hara JA, Vanhulle VP, Delzenne N, Gallez B. Insulin increases the sensitivity of tumors to irradiation: involvement of an increase in tumor oxygenation mediated by a nitric oxide-dependent decrease of the tumor cells oxygen consumption. *Cancer Res.* 2002;62:3555–61.
- Jordan BF, Beghein N, Aubry M, Grégoire V, Gallez B. Potentiation of radiation-induced regrowth delay by isosorbide dinitrate in FSaII murine tumors. *Int J Cancer.* 2003;103:138–41.
- Jordan BF, Sonveaux P, Feron O, Grégoire V, Beghein N, Gallez B. Nitric oxide-mediated increase in tumor blood flow and oxygenation of tumors implanted in muscles stimulated by electric pulses. *Int J Radiat Oncol Biol Phys.* 2003;55:1066–73.
- Sonveaux P, Jordan BF, Gallez B, Feron O. Nitric oxide delivery to cancer: Why and how? *Eur J Cancer.* 2009;45:1352–69.
- Jordan BF, Christian N, Crockart N, Grégoire V, Feron O, Gallez B. Thyroid status is a key modulator of tumor oxygenation: implication for radiation therapy. *Radiat Res.* 2007;168:428–32.
- Gallez B, Jordan BF, Baudalet C, Misson PD. Pharmacological modifications of the partial pressure of oxygen in murine tumors: evaluation using in vivo EPR oximetry. *Magn. Reson. Med.* 1999;42:627–30.

14. Bieron K, Swies J, Kostka-Trabka E, Gryglewski RJ. Thrombolytic and antiplatelet action of xanthinol nicotinate: possible mechanisms. *J Physiol Pharmacol.* 1998;49:241–9.
15. Lehmanne E, Van Der Crone L, Grobe-Einsler R, Linden M. Drug-monitoring study (phase IV) of xanthinol nicotinate (complamin) in general practice. *Pharmacopsychiatry* 1993;26:42–8.
16. Taper HS, Woolley GW, Teller MN, Lardis MP. A new transplantable mouse liver tumor of spontaneous origin. *Cancer Res.* 1966;26:143–8.
17. Gallez B, Baudelet C, Jordan BF. Assessment of tumor oxygenation by electron paramagnetic resonance: principles and applications. *NMR Biomed.* 2004;17:240–62.
18. Sersa G, Cemazar M, Miklavcic D, Chaplin DJ. Tumor blood flow modifying effect of electrochemotherapy with bleomycin. *Anticancer Res.* 1999;19:4017–22.
19. Baudelet C, Ansiaux R, Jordan BF, Havaux X, Macq B, Gallez B. Physiological noise in murine solid tumors using T2*-weighted gradient-echo imaging: a marker of tumour acute hypoxia? *Phys Med Biol* 2004;49:3389–411.
20. Galbraith SM, Maxwell RJ, Lodge MA, Tozer GM, Wilson J, Taylor NJ, Stirling JJ, Sena L, Padhani AR, Rustin GJ. Combretastatin A4 Phosphate has tumor antivascular activity in rat and man as demonstrated by dynamic magnetic resonance imaging. *J Clin Oncol.* 2003;21:2831–42.
21. Ansiaux R, Baudelet C, Jordan BF, Crockart N, Martinive P, DeWever J, Grégoire V, Feron O, Gallez B. Mechanism of reoxygenation after antiangiogenic therapy using SU5416 and its importance for guiding combined antitumor therapy. *Cancer Res.* 2006;66:9698–704.