*Int. J. Cancer:* **103,** 138–141 (2003)


Publication of the International Union Against Cancer

SHORT REPORT

# POTENTIATION OF RADIATION-INDUCED REGROWTH DELAY BY ISOSORBIDE DINITRATE IN FSAII MURINE TUMORS

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**Oxygen deficiency in tumors reduces the efficacy of non-surgical treatment modalities such as conventional radiotherapy and chemotherapy. Since tumor perfusion is directly affected by the vascular resistance to flow of vessels feeding the tumor, vasodilator drugs might be a way to increase tumor blood flow and oxygenation. The effects of nitric oxide (NO) donor administration on tumor oxygenation, perfusion and radiation sensitivity were studied in the FSaII tumor model. Local tumor oxygenation was measured using electron paramagnetic resonance oximetry and a fiberoptic probe, OxyLite. We concomitantly measured the modulation of tumor blood flow by laser Doppler flowmetry. We determined FSaII tumor regrowth delay after isosorbide dinitrate administration and irradiation compared to carbogen breathing before irradiation and with X-rays alone. Administration of the NO donor improved the FSaII tumor $pO_2$ concomitant with an increase in tumor blood flow. We also demonstrated an increase in FSaII tumor radiation sensitivity after isosorbide dinitrate administration, which was similar to the effect of carbogen breathing in the same tumor model. Administration of isosorbide dinitrate could be considered in terms of improvement in tumor blood flow and a possible concomitant increase in accessibility of chemosensitizing agents to the tumor, particularly in terms of modification of the tumor response to irradiation.**


**Key words:** *nitric oxide donor; oxygenation; radiosensitization; isosorbide dinitrate; FsaII tumor*

Cells in experimental and human tumors can become deprived of oxygen through abnormal tumor blood supply and rapid tumor cell growth relative to vascular endothelial cell proliferation. Both oxygen diffusion and oxygen consumption by metabolism in tumor cells contribute to the occurrence of hypoxia. Oxygen deficiency is caused by an insufficient oxygen supply as a result of inadequate tumor perfusion *(diffusion-limited hypoxia)* and fluctuations in red cell flux *(acute hypoxia).*[1,2] The partial pressure of oxygen ($pO_2$) plays important roles in the response of tumors to cytotoxic treatments such as chemotherapy, radiotherapy and photodynamic therapy. The importance of tumor vascular development in relation to the interdependence between tumor growth and angiogenesis is of main concern. Clinical trials utilizing strategies to either inhibit tumor vascular growth or directly attack the tumor vasculature are under way.[3] A different approach to the problem consists in the manipulation of tumor blood flow and oxygen delivery to improve either radio- or chemotherapeutic response. The use of vasoactive agents,[4] modifiers of tumor cell oxygen consumption[5–7] or carbogen breathing,[8,9] which is already used in phase III clinical studies, have improved radiation response.

We previously demonstrated that the nitric oxide (NO) donor isosorbide dinitrate had a dose-dependent effect on transplantable liver tumor (TLT) tumor $pO_2$ and that it was the consequence of an increase in tumor blood flow, as demonstrated by magnetic resonance imaging (MRI).[10] Here, we evaluated the effect of isosorbide dinitrate on a second tumor model, which is well characterized for its response to ionizing radiation.[7,11] Using 2 independent techniques, we monitored tumor $pO_2$ after administration of isosorbide dinitrate to FSaII tumor-bearing mice. We concomitantly measured the modulation of tumor blood flow by laser Doppler flowmetry. We further determined the FSaII tumor regrowth delays after isosorbide dinitrate administration and irradiation in comparison to carbogen breathing before irradiation and with X-rays alone.

## MATERIAL AND METHODS

### *Animal tumor model and treatment*

The syngeneic FSaII tumor model[12] was implanted in the thigh of C3H/He mice. To restrain mice during the experiments, anesthesia was first induced by an i.p. injection of ketamine (80 mg/kg)/xylazine (8 mg/kg) and maintained with ketamine alone (30 mg/kg). Isosorbide dinitrate (Cedocard, 1 mg/ml; Byk Belga, Brussels, Belgium) was diluted in saline (1 mg/40 ml) and administered i.p. at a dose of 0.2 mg/kg. Carbogen (5% $CO_2$/95% $O_2$) breathing (5 l/min) was used as a reference treatment.

### *$PO_2$ and blood flow measurements*

Local tumor oxygenation measurements were carried out using 2 independent techniques: electron paramagnetic resonance (EPR) oximetry and the fiberoptic probe OxyLite. We used OxyFlo probes to assess blood flow inside the tumor. Mice were maintained at 37°C using an infrared lamp and a probe combined with a temperature control unit.

*EPR oximetry.* EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. Charcoal (charcoal wood powder, CX0670-1; EM Science, Gibbstown, NJ) was used as the oxygen-sensitive probe in all experiments. Calibration curves were made by measuring the EPR line width as a function of $pO_2$. For this purpose, the charcoal was suspended in a tumor homogenate and EPR spectra were obtained on a Bruker EMX EPR spectrometer (Karlsruhe, Germany) (9 GHz) at 0–21% $O_2$. Nitrogen and air were mixed in an Aalborg gas mixer (Monsey, NY), and the oxygen content was analyzed using the servomex oxygen analyzer OA540 (Analytic Systems, Brussels, Belgium).[13] Mice were injected in the center of the tumor

Grant sponsor: Belgian National Fund for Scientific Research; Grant number: FRSM 3.4560.00; Grant sponsor: Fonds Joseph Maisin.

B.J. is a research assistant for the Belgium National Fund for Scientific Research.

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Received 30 April 2002; Revised 1 August 2002; Accepted 12 September 2002

DOI 10.1002/ijc.10786

(8 mm diameter) using a suspension of charcoal (100 mg/ml, 50 μl injected, 1–25 μ particle size). EPR measurements were started 2 days after the injection. The tumor under study was placed in the center of the extended loop resonator, the sensitive volume of which extended 1 cm into the tumor mass, using a protocol previously described.[10,14]

*Oxylite/Oxyflo technique.* We used the Oxylite in conjunction with Oxyflo (Oxford Optronix, Oxford, UK) for simultaneously and continuously monitoring tissue blood flow, oxygenation and temperature at the same location.[15,16] Fiberoptic microprobes combining a laser Doppler system, an oxygen sensor and a thermocouple were inserted into the tumor. Data were collected continuously at a sampling frequency of 20 Hz, before and after isosorbide dinitrate administration. Oxylite $pO_2$ measurements are single-point values, and the volume sampled was confined to the sensor tip (230 μm diameter).

*Irradiation and tumor regrowth delay assay*

The tumor-bearing leg was locally irradiated with 16 Gy of 250 kV X-rays (RT 250; Philips, Hamburg, Germany). Mice were anesthetized, and the tumor was centered in a 3-cm-diameter circular irradiation field. When tumors reached 8.0 ± 0.5 mm in diameter, mice were randomly assigned to a treatment group and irradiated. After treatment, tumors were measured every day until they reached a diameter of 16 mm, at which time the mice were killed. A linear fit could be obtained between 8 and 16 mm, which allowed us to determine the time to reach a particular size for each mouse. For each tumor, transversal and anteroposterior measurements were obtained. An average tumor diameter was then calculated.

## RESULTS

*Effect of isosorbide dinitrate on tumor oxygenation*

The 2 techniques we used are intended for continuous measurement of the local $pO_2$ without altering the local oxygen concentration and allow real-time study of the oxygen fluctuations in tissues.

EPR oximetry relies on the oxygen-dependent broadening of the EPR line width of a paramagnetic oxygen sensor implanted in the tumor.[14,17] The fiberoptic OxyLite probes allow $pO_2$ measurement, based on the oxygen-quenched lifetime of a luminescent ruthenium dye.[15,16] We demonstrated previously that the effect of isosorbide dinitrate on TLT tumor $pO_2$ was dose-dependent and that a dose of 0.2 mg/kg was the most efficient.[10] Using the same dose, the basal FSaII tumor $pO_2$ was 3.1 ± 0.1 mm Hg and increased to a maximum value 30 min after injection of isosorbide dinitrate (12.5 ± 1.2 mm Hg, $n = 5$) (Fig. 1). Since the measurement volumes are different for EPR oximetry and the OxyLite probe, individual tumor $pO_2$ values measured with these 2 techniques may be quite different. Nevertheless, mean $pO_2$ values for a group of mice do not differ significantly from one technique to another. Isosorbide dinitrate was proportionally more effective at increasing oxygenation in the FSaII tumor model than on the previously studied TLT tumor model. Carbogen breathing, used as a positive control, induced an increase of 18.7 mm Hg in the FSaII tumor model ($n = 10$).

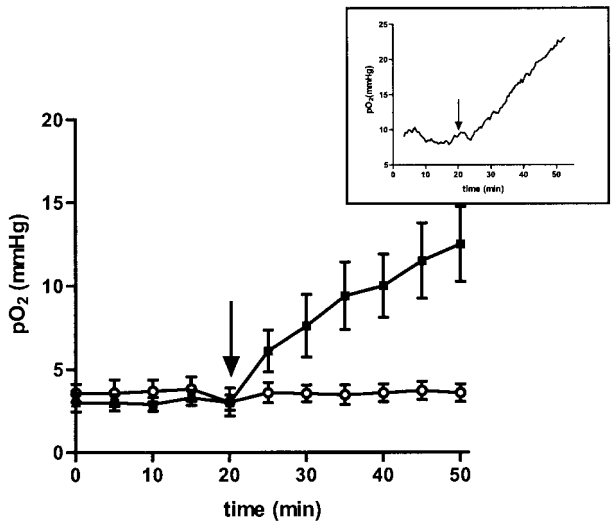


**FIGURE 1** – Effect of isosorbide dinitrate injection on tumor $pO_2$. Graph, mean FSaII tumor $pO_2$ (±SEM) monitored by EPR oximetry ($n = 5$/group) before and after isosorbide dinitrate administration (0.2 mg/kg i.p.). Open circles, control group; solid squares, isosorbide dinitrate group; arrow, time of injection. *(Inset)* Typical FSaII tumor $pO_2$ monitored by OxyLite before and after isosorbide dinitrate administration.

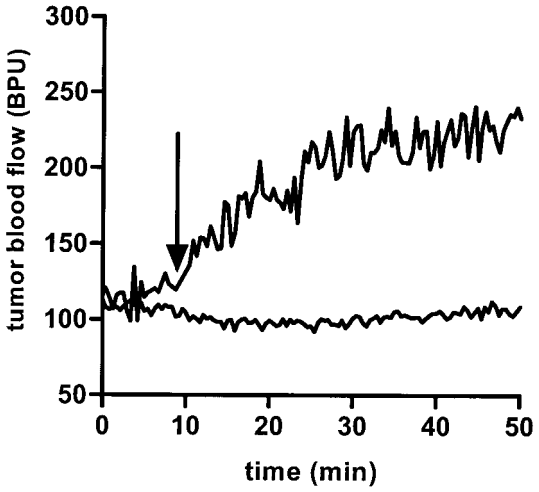


**FIGURE 2** – Effect of isosorbide dinitrate injection on tumor blood flow: typical FSaII tumor blood flow monitored by OxyFlo. Measurements of blood flow are in arbitrary units (BPU, blood perfusion units). Arrow, time of isosorbide dinitrate injection (0.2 mg/kg i.p.).

*Effect of isosorbide dinitrate on tumor blood flow*

We used the OxyFlo technique, based on laser Doppler flowmetry, to assess blood flow inside the tumor, which allows relative measurements of blood flow in arbitrary units (blood perfusion units). We demonstrated a slight increase in tumor blood flow of 34.8% ± 9.5% ($n = 3$) after isosorbide dinitrate administration. However, great variability was observed between results, probably due to the confinement of the measurement volume around the probe and to the inter- and intratumor heterogeneity of tumor vascular networks. A typical experiment is shown in Figure 2.

*Effect of isosorbide dinitrate on sensitivity of tumors to irradiation*

To determine whether isosorbide dinitrate had an effect on the tumor response to radiotherapy, FSaII tumor-bearing mice were treated with irradiation alone or with the combination of isosorbide dinitrate and irradiation and the tumor regrowth delays measured. As the FSaII tumor model is known to be radiosensitized by carbogen,[7,11] we compared the effect of carbogen breathing during irradiation to the effects of isosorbide dinitrate administration. Four groups of 6 mice were used. To avoid tumor cure but still achieve a measurable regrowth delay, a single irradiation dose of 16 Gy was selected as the radiation dose (RX) after preliminary tests. The regrowth delay to reach 12 mm tumor diameter was 5.1 ± 1.1 days for RX alone, 7.4 ± 0.6 days for carbogen and RX ($p < 0.05$) and 7.1 ± 0.4 days for isosorbide dinitrate and RX ($p < 0.05$) (Fig. 3). These data indicate that isosorbide dinitrate increased the sensitivity of the tumor to X-ray irradiation, increasing regrowth delay by a factor of 1.39 compared to a factor of 1.45 for

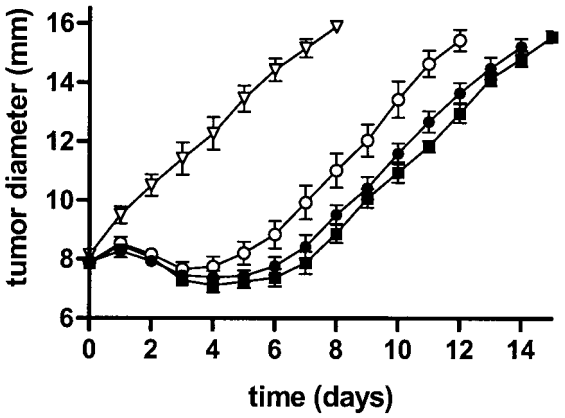


**FIGURE 3** – Effect of the combination of isosorbide dinitrate and radiation on FSaII tumor regrowth. Mice were untreated (open triangles), treated with 16 Gy of RX alone (open circles), treated with isosorbide dinitrate 15 min before 16 Gy of RX (solid circles) or treated with carbogen 15 min before and during irradiation with 16 Gy of RX (solid squares). Each point represents the mean tumor size ± SEM of 6 tumors. Regrowth delays to reach 12 mm tumor diameter were 5.1 ± 1.1 days for RX alone and 7.1 ± 0.4 days for isosorbide dinitrate and RX ($p < 0.05$). Isosorbide dinitrate increased the regrowth delay by a factor of 1.39.

carbogen treatment, which is currently being used successfully in the clinic.[8] Isosorbide dinitrate administration and carbogen breathing have the same efficacy at radiosensitizing FSaII tumors after a single-dose irradiation protocol.

## DISCUSSION

Administration of an NO donor (isosorbide dinitrate) improves FSaII tumor $pO_2$. Our results are in good correlation with our previous experiments performed on the TLT tumor model.[10] The increase in FSaII tumor oxygenation is the result of an increase in tumor blood flow, as demonstrated using OxyFlo. We already showed an increase in tumor blood flow after NO donor administration using Gd-DTPA contrast-enhanced MRI in the TLT tumor model.

We also demonstrated in the present study an increase in FSaII tumor radiation sensitivity after isosorbide dinitrate administration, which was similar to the effect of carbogen breathing in the same tumor model. We can only speculate as to why isosorbide dinitrate is as efficient at radiosensitizing FSaII tumors as carbogen when carbogen induces a greater increase in tumor $pO_2$. Besides the radiosensitizing effect due to oxygen, an additional effect of NO itself cannot be excluded since NO is a radiosensitizer of hypoxic cells *in vitro* and *in vivo.*[18,19]

Administration of isosorbide dinitrate could be considered in terms of improvement in tumor blood flow and a possible consecutive increase in accessibility of chemosensitizing agents to the tumor, particularly in terms of modification of the tumor response to irradiation.

Since tumor perfusion is directly affected by the vascular resistance to flow of vessels feeding the tumor, vasodilator drugs might be a way to increase tumor blood flow and oxygenation. Changes in tumor blood flow caused by external factors are greatly influenced by the structural relationship between vascular beds of tumor and surrounding normal tissues. When the vascular beds in tumors parallel those in normal tissues, changes in perfusion would be opposite: an increase in normal tissue blood flow due to vasodilation would shunt away the blood flow from the tumor to the normal tissue, resulting in a decrease in tumor blood flow *(steal effect).* However, if these vessels are located in series, dilation of host tissue vessels may improve tumor blood flow.[20] In many tumors, series and parallel types may be mixed or combined, and modifications of tumor perfusion vary depending on the relative contribution of these 2 types. Because many tumors lack vascular smooth muscle cells, modifications of tumor blood flow are mainly due to changes in blood flow in adjacent normal tissues. This is the main reason that the potential effect of vasodilators on tumor perfusion is unclear.

The effect of NO donors on the tumor microenvironment was previously investigated by several groups. Wood *et al.*[21] described an improvement of the bioenergetic status and the radiosensitivity of experimental tumors after application of the NO donor SIN-1. We also found that several NO donors (molsidomine, sodium nitroprusside, nitroglycerin and isosorbide dinitrate) increased $pO_2$ in TLT tumors.[10,14] On the contrary, Thews *et al.*[22] described decreased tumor perfusion in rats bearing s.c. DS sarcomas, which correlated with a fall in mean arterial blood pressure, resulting in a decrease in red blood cell flux by ±40%. Although contradictory, these results lead to an interesting question concerning the level of NO effectively delivered to the tumor. Different NO donors may have different efficacy and kinetics in terms of NO delivery. Moreover, the i.p. injection we performed for administration of isosorbide dinitrate does not guarantee the same dose in the blood circulation and should induce only a short increase in NO levels compared to the 30 min i.v. infusion used by Thews *et al.*[22] Like the opposite effects described with different doses of hydralazine administration, NO donors could have different actions at high and low doses.

A likely additional effect of isosorbide dinitrate on blood rheology should also be considered. Indeed, the effect of nitrovasodilators on blood viscosity has already been studied *in vitro* and *in vivo.* Both blood cell shape and blood viscosity are influenced *in vitro* by some nitrovasodilators.[23,24] This effect could be important by decreasing the transient occlusion of vessels (acute hypoxia).

Finally, we previously found that NO can also modulate the $pO_2$ in tumors by decreasing the oxygen consumption of tumor cells after insulin infusion through regulation of mitochondrial respiration.[7]

In conclusion, we found that isosorbide dinitrate increased $pO_2$ and blood flow in FSaII tumors. This effect induced a radiosensitizing effect on the tumor. Thus, appropriate modulation of NO levels in tumors may lead to enhancement of the response of tumors to irradiation.

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