Validation of the Fluorinated 2-Nitroimidazole SR-4554 as a Noninvasive Hypoxia Marker Detected by Magnetic Resonance Spectroscopy

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ABSTRACT

Purpose: Tumor hypoxia is associated with poor prognosis and a more malignant tumor phenotype. SR-4554, a fluorinated 2-nitroimidazole, is selectively bioreduced and bound in hypoxic cells. We present validation studies of SR-4554 as a noninvasive hypoxia marker detected by fluorine-19 magnetic resonance spectroscopy (19F MRS) in the P22 carcinosarcoma, a tumor with clinically relevant hypoxia levels.

Experimental Design: Tumor-bearing female severe combined immunodeficient mice received SR-4554 at 180 mg/kg. Pharmacokinetic studies of parent SR-4554 in plasma and tumors were performed using high-performance liquid chromatography-UV. Total SR-4554 (parent SR-4554 and bioreduction products) was monitored in tumor by 19F MRS using a 4.7 T spectrometer, with continuous acquisition for up to 5 h. A parameter of total SR-4554 retention, the 3-h 19F retention index (19FRI) was determined. Tumor pO2, assessed polarographically, was decreased (5 mg/kg hydralazine or 100 mg/kg combretastatin A-4 phosphate) or increased [1 l/min carbogen (5% CO2, 95% O2) plus 500 mg/kg nicotinamide], and the corresponding 19FRI was measured.

Results: Comparative HPLC-UV- and MRS-derived assessments of parent and total SR-4554, respectively, indicated that concentrations of total SR-4554 consistently exceeded parent SR-4554, the differential increasing with time. This indicates formation and retention of SR-4554 bioreduction products in tumor, confirming the presence of hypoxia. The 19FRI was higher in hydralazine- and combretastatin-treated animals compared with unmodulated animals (P = 0.004 and 0.15, respectively) and animals receiving carbogen and nicotinamide (P = 0.0001 and 0.005, respectively). Significant correlations were demonstrated between mean 19FRI and polarographic pO2 parameters (P < 0.002).

Conclusions: Retention of hypoxia-related SR-4554 bioreduction products can be detected in the clinically relevant P22 tumor by 19F MRS, and the 19FRI correlates with polarographically measured pO2. These findings support the use of SR 4554 as a noninvasive hypoxia marker.

INTRODUCTION

This report describes laboratory studies that underpin the clinical development of the hypoxia marker SR-4554 (Fig. 1; Refs. 1, 2).

Tumor hypoxia has long been recognized as a cause of radioresistance and radiotherapy treatment failure (3). In clinical studies, tumor oxygenation has been related to poor treatment outcome in a number of tumor sites (4–9). At the molecular level, hypoxia induces the transcription factor hypoxia-inducible factor-1α, resulting in expression of a range of genes that participate in the normal adaptive response of the cell to hypoxia (10, 11). In addition, cells lacking wild-type p53 are unable to undergo hypoxia-induced apoptosis (12) and are consequently conferred with a survival advantage, leading to genetic instability and enhanced tumor progression (13). Thus, molecular evidence is emerging to support clinical observations, indicating that tumor hypoxia has wide-reaching implications.

It is possible to modify tumor hypoxia and thereby improve response to radiotherapy by strategies such as hyperbaric oxygen, radiosensitizers, and carbogen and nicotinamide (14). Furthermore, hypoxia, as a feature distinguishing the tumor microenvironment from that of normal tissues, can be exploited via bioreductive cytotoxic agents and gene-directed enzyme prodrug therapy approaches using hypoxia-responsive genes (15). To select patients who are most suitable for such interventions, it is important to be able to measure tumor hypoxia by a simple, noninvasive, and routinely applicable technique. Such a technique could also be useful for monitoring the effects of therapies that modulate tumor hypoxia, for example antivascular drugs.
Validation of Fluorinated 2-Nitroimidazole SR-4554

Nitroimidazoles are bioreductive agents that undergo 1-electron reduction by intracellular reductases selectively under hypoxic conditions to generate reactive intermediates that become covalently bound to cellular constituents (18). These retained bioreduction products, therefore, indicate the presence of cellular hypoxia. Nitroimidazoles have been used extensively in the past as hypoxic cell radiosensitisers (19) and more recently have gained a role as markers of tumor hypoxia. Methods of detection include immunohistochemistry, e.g., EF5 (20) and pimonidazole (21), and noninvasive scanning techniques such as positron emission tomography, e.g., 18F-fluoromisonidazole (22), single photon emission computerized tomography, e.g., 151I-IAZA (23), and MRS, e.g., CCI-103F (24, 25). SR-4554, a new fluorinated 2-nitroimidazole, has been designed specifically for use as a noninvasive hypoxia marker detected by 19F MRS and has already undergone preclinical evaluation in vitro and in vivo (1, 2, 26–29). An important feature of SR-4554 is its favorable pharmacokinetic properties, in particular exclusion from the nervous system, thereby reducing the potential for neurotoxicity (27), a limiting toxicity of nitroimidazole radioisotopes (19) and has already undergone preclinical evaluation. To identify cellular hypoxia, it is necessary to measure the generation and accumulation of hypoxia-dependent SR-4554 bioreduction products in tumor. This has been achieved in mice by comparing 18F signal from retained bioreduction products acquired at a late (6-h) time point, with signal acquired at an early (45-min) time point when parent SR-4554 peaks in tumor (1). This ratio, the 19FRI, has been shown to correlate with the corresponding radiobiological hypoxic fraction for four murine tumors (1).

These earlier findings established initial proof of principle for the use of SR-4554 as a hypoxia marker. We now describe further validation studies of SR-4554 in tumor-bearing animals that are considerably more extensive than previous investigations in animal models (1, 29). In addition, the present studies use the P22 carcinosarcoma, a rat tumor that has an oxygenation profile (30) consistent with that encountered in many human tumors in patients (7, 8, 31), in terms of both higher absolute tumor pO2 levels and greater heterogeneity of intratumor oxygenation vis-à-vis many rodent tumor models (32). It is therefore more representative of the clinical scenarios in which SR-4554 will eventually be used as compared with the more homogeneous, hypoxic tumor models used previously (1, 29). In further contrast with previous work, the present experiments were carried out without the complications of administered anesthetics.

The overall objectives of the current study were 3-fold: (a) to demonstrate SR-4554 retention in the clinically relevant P22 tumor; (b) to produce a parameter of SR-4554 retention that reflects tumor hypoxia and to provide validation for its use as a diagnostic assay; and (c) to evaluate the ability of this parameter to measure tumor oxygenation by comparison with a more standard technique, a polarographic electrode. Specifically, the initial aims were to fully evaluate the behavior of SR-4554 in P22 tumor-bearing animals and to characterize the time course for retention by tumor of SR-4554 bioreduction products. Having successfully demonstrated SR-4554 retention by the P22 tumor, the next aims were to calculate the 19FRI as the chosen parameter of tumor hypoxia and to demonstrate it to be robust over a wide dynamic pO2 range, generated by the use of a selection of agents that modulate tumor oxygenation. The use of a wide pO2 range was also of clinical relevance because although human tumors are on average less hypoxic than rodent models, human tumors with extreme pO2 profiles, both hypoxic and oxic, are sometimes encountered (33, 34). The final aim was to correlate the manipulated 19FRI with pO2 measured directly using a polarographic electrode. The results obtained in the current study support the ongoing clinical development of SR-4554 as a hypoxia marker. This is particularly important because SR-4554 has now entered clinical study at the Royal Marsden Hospital and Institute of Cancer Research under the auspices of Cancer Research UK in association with SRI International (Menlo Park, CA).

MATERIALS AND METHODS

Drugs and Reagents. SR-4554 was synthesized and purified at SRI International and was kindly provided by Dr. Andrew B. Kelson. It was formulated as a 6 mg/ml solution in 0.9% saline and 2.7% DMSO (Sigma-Aldrich Co., Dorset, United Kingdom) for use in vivo and was administered in all experiments i.p. at 180 mg/kg. The MRS 19F external standard FTrp was acquired at Sigma-Aldrich. The MRS 2H external standard TSP was acquired from Wilmad (Buena, NJ). Pimonidazole (Hydroxyprobe-I) was obtained from Natural Products International, Inc. (Research Triangle Park, NC). Hydralazine and nicotinamide were obtained from Sigma-Aldrich. Combretastatin A-4 phosphate was synthesized at the Gray Cancer Institute by a published method (35). Carbogen gas (95% O2, 5% CO2) was obtained from British Oxygen Company (Guildford, United Kingdom). The internal standard used in the HPLC-UV analysis, 1-(2-nitro-1-imidazolyl)-3-chloro-2-propyl alcohol (Ro 07-0269), was obtained from Roche Products (Welwyn City, Herts, United Kingdom). All other reagents were of HPLC or analytical reagent grade.

Animals and Tumor Models. The P22 rat carcinosarcoma arose in the treated site of a male BD9 rat after spinal cord irradiation in the cervical region (36). In the present study, the tumor was implanted and grown s.c. as a xenograft in the lower back of female SCID mice. It was maintained by serial transplantation of 4–6 × 105 cells, harvested by enzymatic disaggregation for up to 12 passages, followed by return to definitive frozen stock. Tumors were typically used at a mean (±2 SD) diameter of 10.7 (±0.6) mm, including skin thickness. Animals were restrained unanesthetized within Lucite jigs.

Experimental protocols were approved by the Gray Cancer Institute Ethical Review Committee and the Institute of Cancer Research.

Figure 1: Chemical structure of SR 4554.
Research Ethics Committee. All mice were maintained and used according to national and local codes of practice for the housing and care of animals used in the scientific procedures, including the United Kingdom Co-ordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia (37).

Assessment of Tumor Hypoxia with Pimonidazole. Pimonidazole hydrochloride, available commercially as an immuno-histochemical marker of tissue hypoxia (Hypoxyprobe-1), was used to establish that the P22 tumor can bioreduce and bind 2-nitroimidazole drugs. Pimonidazole (60 mg/kg i.p.) was administered to tumor-bearing animals. One group of 10 animals was killed by cervical dislocation 90 min later, as described previously (38). A second group of 10 animals received hydralazine (5 mg/kg i.p.) 15 min after pimonidazole to increase tumor hypoxia. These animals were killed 5.75 h later. The plasma elimination half-life of pimonidazole in C3H mice is 31.6 min (39). Assuming a similar value in SCID mice, at the time of killing (90 min after administration) plasma levels will have fallen by nearly 90%, thereby minimizing generalized background staining by residual pimonidazole when tumors become hypoxic after excision. However, hydralazine is known to reduce renal perfusion and glomerular filtration rate (40), thus delaying clearance of renally eliminated drugs (such as pimonidazole), with the effect of increasing the plasma half-life. The time to killing in hydralazine-treated animals was therefore increased to ensure sufficient time for elimination of circulating drug from plasma. Tumors were removed immediately after killing, fixed in formalin, and embedded in paraffin.

Four μm sections were cut, and tissue antigens were detected using the commercially available kits Hypoxyprobe-1 and UltraVision Mouse Tissue Detection System: Anti-Mouse, HRP/DAB (LabVision Corp., Tremont, CA). Kits were used as directed by the manufacturers and according to a published method (41).

One immunostained section per tumor was viewed at ×50 using an Axioskop microscope (Zeiss, Jena, Germany) with a video zoom adapter and ×5 objective. Images (field size, 768 × 576 μm) of the entire tumor section (mean number of images per tumor, 32; range, 21–47) were captured and digitized by a high resolution video camera (JVC KY-F55B, Japan) linked to a high resolution color monitor and Visilog computer software (version 5.0; Noesis Vision, Inc., Montreal, Quebec, Canada). Computer software developed in-house at the Gray Cancer Institute was used to delineate and quantify stained areas on captured images. Pimonidazole binding was recorded as the percentage of immunostained area in the total tumor area, summing all fields for individual tumors.

Pharmacokinetic Profile of Parent SR-4554 in Tumor and Plasma by HPLC-UV Analysis. To evaluate the pharmacokinetic profile of parent SR-4554 in the P22 tumor and corresponding mouse plasma, tumor-bearing animals received SR-4554 and were placed in Lucite jigs to reproduce conditions of animals undergoing MRS. Animals were sacrificed by cervical dislocation at 0.5, 0.75, 1, 2, 3, and 4.5 h after SR-4554 administration (3–7 animals per time point). Collection and storage of plasma and tumor samples were carried out as described above.

SR-4554 was extracted from tumor and plasma samples and was measured by HPLC-UV analysis. Duplicate standard curves were prepared in the matrix of origin (plasma or tumor homogenate) by spiking with external SR-4554 at levels of 0.1, 0.4, 1, 4, 10, 40, 100, and 160 μg/ml. Duplicate quality control samples were included at levels of 1.2, 20, and 120 μg/ml. Samples were prepared by spiking 250 μl of plasma or tumor homogenate (containing 50 mg tumor in Tris-HCl buffer) with 30 μl of internal standard (50 μg/ml) and adding 10 μl of 10% HCl and 1 ml of acetonitrile. After centrifugation for 2 min at 1300 rpm, the organic phase was removed and dried, and the residue was dissolved in 100 μl of methanol. Ten-μl aliquots were injected onto the HPLC-UV system, which consisted of an AS3000 auto-sampler, P4000 pump, and UV6000 detector (ThermoQuest, Hemel Hempstead, United Kingdom). Separation was achieved on a 15-cm column (ABZ + 5 μm, 4.6 mm inside diameter; Supelco, Poole, Dorset, United Kingdom) with a gradient of methanol in 30 mM ammonium acetate over 15 min (5–15% methanol over 10 min, 15–100% over 15 min) at flow rate of 1 ml/min at 40°C. Detection was by UV at 324 nm.

All data collection and processing were performed using a ChromQuest Chromatography Data System (ThermoQuest, Inc., San Jose, CA) associated with the HPLC-UV system. The UV response (peak area ratio of analyte to internal standard) of each calibration standard was plotted against the nominal concentration to produce a standard curve, shown by least squares regression analysis to be linear (r = 0.999). Five of the six quality control samples were required to fall within 15% of nominal concentrations. Pharmacokinetic data from HPLC and MRS were analyzed by noncompartmental analysis using Pharsight WinNonLin Version 3.0 computer software (Pharsight Corp., Mountain View, CA).

The assay detects parent SR-4554 but not bioreduction products, because the latter lack the nitro chromophore that is responsible for the absorption of SR-4554 at 324 nm (26). Plasma SR-4554 content was expressed as micrograms per milliliter of plasma (μg/ml), and tumor content was expressed as micrograms per gram of tumor (μg/g). These units were assumed to be directly comparable on the basis of 1 ml of water weighing 1 g, and a tumor water content of 85%.

In Vivo MRS Protocol. Tumor-bearing mice received injections of SR-4554 and were restrained in Lucite jigs. A 13-mm diameter, double-tuned 19F/1H surface coil was fitted over the tumor, and animals were inserted into a horizontal bore 4.7 T (200 MHz for 1H) magnetic resonance spectrometer (UnityInova, Varian, Palo Alto, CA). An 18-μl volume reference bulb containing FTRp and TSP was placed adjacent to the tumor inside the surface coil to act as a chemical shift reference and to enable quantification of the 19F signal. Shimming was performed on the proton (1H) resonance of water using the same
transmitter-receiver coil tuned to the $^1$H resonance frequency (200 MHz). Typically, a line-width of 40 Hz was obtained. The spectrometer was tuned to $^2$H (30 MHz). Signal was acquired using a pulse-acquire sequence with a TR (time to repetition) of 1 s in two blocks of 160 averages with a pulse width of 40 µs equivalent to an approximate 90° flip angle at the reference bulb and a spectral width of 2 kHz. The spectrometer was then tuned to $^{19}$F (188 MHz), and signal was acquired continuously for up to 4 h using a pulse-acquire sequence with a TR of 4 s in blocks of 64 averages (with a pulse width of 60–100 µs calibrated for each tumor to give a 90° flip angle at the reference bulb and a spectral width of 20 kHz). Final $^{19}$F tuning was checked; the spectrometer was then returned to $^1$H, and signal was acquired as before. Pulse repetition times were at least three times the $^{19}$F longitudinal ($T_1$) relaxation time ($\pm$SE) of SR-4554 (1.23 ± 0.2 s) and FTrp (1.13 ± 0.07 s), and the $^2$H $T_1$ values ($\pm$SE) of natural abundance deuterium in water (HOD, 0.34 ± 0.03 s) and in TSP (0.05 ± 0.005 s). All $T_1$ values were measured $in vitro$ using an inversion recovery sequence with at least seven inversion times and a three-parameter least-squares fitting procedure (from the Varian software).

Data analysis was performed using the VARPRO (42) time-domain fitting with prior knowledge algorithm of MRUI (Magnetic Resonance User Interface) version 97.2 (43) within Matlab version 4.2c (The Mathworks, Natwick, MA). For each tumor, $^{19}$F signal line-widths were estimated to be constant throughout the time course. In the case of $^1$H spectra, the two signals from TSP were assumed to be of equal line-width and intensity and to have a fixed frequency difference. All signals were fitted with a fixed phase. Initial values for signal frequencies were input from observation of the spectra, but no further assumptions of prior knowledge were made. In addition to estimates of signal amplitudes, VARPRO also provides error estimates on those amplitudes. This information was used to evaluate the quality of each spectrum.

**Absolute Quantification of SR-4554 Detected by $^{19}$F MRS.** Previous work has shown that although $in vitro$ studies to detect SR-4554 have identified two distinct $^{19}$F peaks (from parent SR-4554 and bioreduction products, respectively), the chemical shift separation between them is only 0.12 ppm, which is insufficient for $in vivo$ resolution (28). Hence, a single $^{19}$F peak, representing a composite of signal from parent SR-4554 and bioreduction products (total SR-4554), is acquired during $in vivo$ $^{19}$F MRS (1). Absolute concentration of this total SR-4554 in tumor was estimated using a method developed previously (1) and derived from the method of Thulborn and Ackerman (44). This uses a double-tuned $^{19}$F/$^1$H circuit, comparing $^{19}$F signal from the tumor (SR-4554) with fluorine signal from the reference bulb (FTrp), and comparing deuterium (HOD) signal from tumor water with deuterium signal from the reference bulb (TSP). The following equation was used:

$$[SR-4554]_{tumor} \text{ (mm)} = \frac{SI_{SR-4554} \times SI_{TSP}}{SI_{FTrp} \times SI_{HOD}}$$

where $SI_{SR-4554}$, $SI_{TSP}$, $SI_{FTrp}$, and $SI_{HOD}$ are the integrated signal intensities of SR-4554, FTrp, TSP, and HOD, respectively. $F$ is a factor that depends on the concentration of internal and external standards, the number of magnetically equivalent $^2$H atoms/molecule of TSP, and the number of magnetically equivalent $^{19}$F atoms/molecule of SR-4554. The concentration of HOD in tumor was calculated on the basis that the natural abundance of deuterium in water is 0.0156% (45). The molarity of hydrogen in pure water is 111.1 g/l, giving a concentration of deuterium in water of 17.33 mM. Assuming tumor water content of 85%, the tumor concentration of HOD is 14.73 mM. The method was validated in triplicate in 540-µl glass phantoms using solutions of SR-4554 ranging from 22.6–361.0 µg/ml (0.04–1.28 mM) prepared in 0.9% saline. Results were expressed as micrograms of SR-4554 per milliliter of tumor ($\mu$g/ml) to allow direct comparison with results obtained by HPLC analysis.

**Measurement of $^{19}$F Retention in Tumors.** Retention of $^{19}$F, representing total SR-4554 (defined as parent SR-4554 and bioreduction products), was quantified as a parameter termed the $^{19}$FRI. This was the ratio of total SR-4554 concentrations determined by MRS at a later time point (2, 3, or 4 h) relative to an early time point (0.75–1 h) after administration of SR-4554. The early time point was selected as the time of peak concentration of parent SR-4554 in tumor, as established by tumor pharmacokinetic studies. Measurements at later time points comprised any residual parent SR-4554 still present in tumor together with retained SR-4554 bioreduction products.

All total SR-4554 concentrations were calculated by averaging three consecutive blocks of 8.5 min of $^{19}$F signal acquisition (for the early time point, these were the three blocks closest to 45–60 min; for the 2, 3, and 4 h time points, these were the blocks closest to the chosen time point together with one block on either side).

**Effect on $^{19}$FRI of Agents That Modulate Tumor Oxygenation.** Modulatory agents were used to increase (carbogen plus nicotinamide) or decrease (hydralazine or combretastatin A-4 phosphate) tumor oxygenation. Carbogen and nicotinamide used in combination significantly decrease tumor hypoxia (46, 47). Used together, the two agents have been shown to effectively and consistently increase tumor oxygenation in the P22 tumor for up to 4 h when measured by polarographic electrode (data not shown). Nicotinamide (500 mg/kg i.p.) was administered immediately after SR-4554 injection. Animals were then placed in Lucite jigs, and carbogen gas commenced at a flow rate of 1 l/min. Gas flow was continued throughout spectroscopy measurements for a total duration of 4 h.

Hydralazine at a dose of 5 mg/kg produces decreased tumor perfusion (40, 48) and increased tumor hypoxia (49, 50). This dose has been shown by polarographic electrode studies in the P22 tumor to produce a significant and sustained increase in tumor hypoxia from 15 min for up to 4.5 h after hydralazine administration, $i.e.$, for the duration of MRS studies (data not shown). Hydralazine (5 mg/kg i.p.) was therefore administered 15 min after SR-4554 injection to ensure adequate penetration of tumor by SR-4554 before onset of any generalized hypotension and its resultant effects on tumor perfusion.

CA-4-P is a tubulin-binding agent structurally similar to colchicine. It is cytotoxic toward proliferative endothelial cells (16) and rapidly destabilizes the endothelial cytoskeleton (51). It has been shown to induce rapid and marked increases in tumor vascular resistance and selective decreases in tumor blood flow (52, 53). It has also been shown to produce significant increases.
in tumor hypoxia measured by polarographic electrode within 30 min, which were sustained for at least 6 h (54). CA-4-P (100 mg/kg i.p.) was administered 30 min after SR-4554 injection to ensure that sufficient SR-4554 had reached tumor before the onset of shut down of tumor vasculature.

Tumor Oxygenation Measurements by Polarographic Electrode. $pO_2$ measurements in tumors were performed using a fine needle oxygen polarographic electrode (KIMOC-6550; Eppendorf, Hamburg, Germany; Refs. 55, 56) immediately after spectroscopic measurements were completed (~4 h after SR-4554 administration). Between six and nine discrete tracks were made in each tumor, each track at a different angle. For each track, the electrode was inserted to a depth of at least 1 mm, and $pO_2$ readings were allowed to stabilize; the electrode was then advanced automatically through the tissue in forward (0.9-mm) increments followed by backward (0.3-mm) steps before recording a $pO_2$ reading. Approximately 90 (range, 45–105) readings were taken within each tumor. After final electrode removal, a thermocouple was inserted to measure the temperature of the tumor center. Atmospheric pressure was recorded. Readings were then corrected by the data processing unit of the device for variations in tumor temperature and atmospheric pressure. Probe calibration was carried out before and after in situ measurements and was repeated at least every 90 min. Any tracks containing more than one $pO_2$ value calculated as less than ~2 mm Hg were excluded from analysis because of the possibility of pressure artifact producing spuriously low $pO_2$ readings (30). Results were expressed as median $pO_2$ and percentage of $pO_2$ values <2.5, 5, or 10 mm Hg.

Statistical Methods. Differences between two groups were tested for significance using a Student’s $t$ test for unpaired data. Differences between more than two groups were tested for significance using an ANOVA (one-way ANOVA), followed by Fisher’s post-hoc least significant difference test. Calibration curves were produced using linear regression analysis. Correlations were analyzed by Pearson correlation calculations. Statistical significance was defined as $P < 0.05$. All $P$s were two-sided.

RESULTS

Pimonidazole Binding

Pimonidazole binding was used as an established method for detecting tumor hypoxia (21, 38, 57). The mean ± SE percentage of immunostained area in the total tumor area was 1.5 ± 0.5% and 37.3 ± 5.2% for groups of unmodulated and hydralazine-treated animals, respectively ($P < 0.0001$). This indicates that the P22 tumor is capable of bioreducing and binding nitroimidazole drugs because pimonidazole binding is dependent on bioreduction (18). The results are consistent with a relatively low level of intrinsic hypoxia in the P22 tumor, which was increased considerably by hydralazine.

Pharmacokinetic Profile of Parent SR-4554 in Plasma and P22 Tumor

The plasma and tumor pharmacokinetics of parent SR-4554 in unmodulated tumor-bearing female SCID mice are shown in Fig. 2 and Table 1. The drug was rapidly absorbed, with the highest plasma concentration ($C_{\text{max}}$, 324.8 μg/ml) observed at 30 min, the earliest time point examined. Clearance from plasma was rapid with a half-life of 37 min, such that by 3 h, parent SR-4554 had fallen to 2% of $C_{\text{max}}$, and by 6 h was approaching the lower limit of quantification of the assay (0.1 μg/ml). SR-4554 peaked in tumor between 45 and 60 min ($C_{\text{max}}$, 58.3 μg/ml). As in plasma, clearance was rapid with a half-life of 39 min. By 3 h, parent SR-4554 had fallen to 10% of $C_{\text{max}}$, and by 6 h was almost undetectable. Tumor:plasma ratios of parent SR-4554 were highest at 2 and 3 h, with values of 0.81 and 0.85, respectively.

Pharmacokinetic Profile of Total SR-4554 Detected by $^{19}$F MRS

Fig. 3 shows typical $^{19}$F and $^2$H spectra obtained from a P22 tumor, 60 min after the tumor-bearing mouse received SR-4554. Deuterium spectra were acquired to enable quantification of total SR-4554 $^{19}$F signal, from parent SR-4554 and bioreduced products, in tissues. The lower limit of detection by $^{19}$F MRS was 22.6 μg/ml. This was determined by the criterion that the computer-fitted SR-4554 peak intensity was >4 times the error estimate (the SD error determined from the VARPRO procedure). Regression analysis comparing SR-4554 concentrations obtained by $^{19}$F MRS with the corresponding nominal concentrations demonstrated the calibration curve to be linear over the range 22.6–361.0 μg/ml ($r = 0.96$; slope = 0.96; $P < 0.0001$). Tumor pharmacokinetics of total SR-4554 detected by $^{19}$F MRS in unmodulated animals are shown in Fig. 2 and Table 1, alongside the corresponding HPLC-UV data for parent SR-4554. Peak concentrations were observed at 45 and 60 min. By 3 h, total SR-4554 had declined to 34% of the highest observed value. Beyond 4 h, $^{19}$F signal was of insufficient intensity to be reliably distinguished from baseline noise.

Comparison of concentrations in tumor of parent SR-4554 measured by HPLC-UV and total SR-4554 detected by $^{19}$F MRS, shows that total SR-4554 exceeded parent SR-4554 at all time points (Fig. 2; Table 1). Up to 1 h the ratio of parent:total SR-4554 was 0.6, indicating that parent SR-4554 is contributing ~60% of the $^{19}$F signal acquired from tumor. Total SR-4554 exceeded parent SR-4554 by ~30 μg/ml, giving an approximate
estimate of the concentration of bioreduction products present in tumor at that time. Beyond 1 h, the ratio of parent:total SR-4554 decreased such that by 3 h, parent SR-4554 contributed only ~18% of the $^{19}$F signal acquired from tumor.

**Calculation of $^{19}$FRI**

$^{19}$FRIs ($\pm$ SE) calculated for 2-, 3-, and 4-h time points were 0.64 ± 0.06, 0.43 ± 0.06, and 0.29 ± 0.04, respectively, for a group of 15 unmodulated animals. The $^{19}$FRI decreased with time because of the declining contribution of parent SR-4554 to the acquired $^{19}$F signal as it washed out of the tumor. A wide range of $^{19}$FRI values was observed between individual tumors at each time point (coefficients of variation were 34, 54, and 54% for 2, 3, and 4 h $^{19}$FRIs, respectively), most likely reflecting the marked degree of intertumor heterogeneity in the P22 tumor (30).

Because the presence of parent SR-4554 contributes to $^{19}$F signal and therefore to measurement of total SR-4554, it is preferable to measure the $^{19}$FRI when substantial washout of the parent drug from the tumor has occurred. The 3-h $^{19}$FRI was judged to be the most reliable parameter of retained SR-4554 bioreduction products and therefore of tumor hypoxia, because this was the time of the lowest ratio of parent:total SR-4554 in tumor (Fig. 2; Table 1) before the concentration of total SR-4554 fell below the limit of detection (22.6 μg/ml).

**Effect of Agents That Modulate Tumor Oxygenation on the Pharmacokinetics of SR-4554 and the $^{19}$FRI**

Carbogen and Nicotinamide. Concentrations of total SR-4554 in tumor as measured by $^{19}$F MRS were slightly higher in animals treated with carbogen and nicotinamide compared with unmodulated animals at all time points (Fig. 4A), although the differences were not statistically significant except at 2 h ($P = 0.14, 0.03, 0.72,$ and 0.10 at 1, 2, 3, and 4 h, respectively). The elimination half-life of total SR-4554 from tumor (81 min)
was marginally shorter than that in unmodulated animals (87 min). The 3-h \(^{19}\)FRI (mean \pm SE, 0.30 \pm 0.03) was lower in carbogen and nicotinamide-treated compared with unmodulated animals, although the difference did not reach statistical significance \((P = 0.15; \text{Fig. 5})\).

Hydralazine. Comparing concentrations of total \(\text{SR-4554}\) measured by \(^{19}\)F MRS in tumors of hydralazine-treated animals with those in unmodulated animals, a number of differences are apparent (Fig. 4B; Table 1). Total \(\text{SR-4554}\) in tumor was present at consistently higher concentrations in hydralazine-treated animals at all time points, the peak tumor concentration was later at 1–2 h, and the elimination half-life of total \(\text{SR-4554}\) from tumor was longer at 112 min. The 3-h \(^{19}\)FRI in hydralazine-treated animals (mean \pm SE, 0.70 \pm 0.08) was significantly higher than that in both unmodulated animals \((P = 0.004)\) and those modulated with carbogen and nicotinamide \((P = 0.0001; \text{Fig. 5})\).

Hydralazine is known to decrease renal perfusion and glomerular filtration rate in experimental animals (40). \(\text{SR-4554}\) is renally excreted, with 70% of parent drug appearing unchanged in the urine (27). It is therefore possible that the observed effects on \(\text{SR-4554}\) pharmacokinetics may be attributable to the potential delay of parent \(\text{SR-4554}\) clearance by hydralazine. This was confirmed by HPLC-UV studies in hydralazine-treated animals, which demonstrated that hydralazine does affect the pharmacokinetic profile of parent \(\text{SR-4554}\) in both plasma and tumor. Parent \(\text{SR-4554}\) peaked in plasma both later (at 1 h) and at a lower concentration in hydralazine-treated compared with unmodulated animals (Fig. 6A; Table 1). Beyond 1 h, parent \(\text{SR-4554}\) was cleared from plasma at a slower rate, until by 4.5 h, 90% had been eliminated. Parent \(\text{SR-4554}\) peaked in tumors of hydralazine-treated animals later (at 2 h) and at a higher concentration \((P < 0.0001)\) than in unmodulated animals (Fig. 6B; Table 1). After 2 h, parent \(\text{SR-4554}\) was cleared at a rate similar to that in plasma such that 84% had been eliminated by 4.5 h. However, at 3 h (the time point used for the calculation of the \(^{19}\)FRI), there were higher concentrations of parent \(\text{SR-4554}\) present in tumors of hydralazine-treated compared with unmodulated animals.

Comparing concentrations of total and parent \(\text{SR-4554}\) in tumors of hydralazine-treated animals, total \(\text{SR-4554}\) was present at consistently higher concentrations than parent \(\text{SR-4554}\) at all time points (Fig. 6C). As in unmodulated animals (Fig. 2), the ratio of parent:total \(\text{SR-4554}\) decreased with time, as demonstrated by the divergence of the respective curves. This indicates the decreasing contribution of parent \(\text{SR-4554}\) to \(^{19}\)F signal acquired from tumor, although at 3 h parent \(\text{SR-4554}\) is contributing to \(^{19}\)F signal to a greater extent than in unmodulated animals. Nonetheless, at 3 h the concentration of total \(\text{SR-4554}\) exceeded that of parent \(\text{SR-4554}\) by a large margin (67 \(\mu\)g/ml), implying a major contribution made to \(^{19}\)F signal by the presence in tumor of \(\text{SR-4554}\) bioreduction products. This is
further indicated by an increase in the ratio of bioreduced SR-4554 (total, parent SR-4554 at 3 h) to the area under the parent SR-4554 tumor concentration versus time curve (0–3 h) from 0.27 in unmodulated tumors to 0.42 in those treated with hydralazine. CA-4-P. Concentrations of total SR-4554 in tumor as measured by 19 F MRS were consistently higher in CA-4-P-treated compared with unmodulated animals at all time points (Fig. 4C). The differential between concentrations in modulated and unmodulated animals became progressively greater between 1 and 4 h, reflected by a prolonged elimination half-life for total SR-4554 from tumor of 130 min. The 3-h 19FRI in CA-4-P-treated animals (mean ± SE, 0.59 ± 0.06) was higher than that in unmodulated animals (P = 0.10) and significantly higher than that in animals modulated with carbogen and nicotinamide (P = 0.005; Fig. 5).

Correlation of 19FRI with Tumor pO2 Parameters Measured by Polarographic Electrode

Mean values for the 3-h 19FRI were compared with the corresponding mean pO2 values measured polarographically in groups of unmodulated animals and in those modulated by carbogen and nicotinamide, hydralazine, or CA-4-P (Fig. 7). Highly significant correlations were obtained between the mean 19FRI and the mean values for the more stringent parameters of hypoxia, i.e., % pO2 values <2.5 mm Hg (r = 0.63, P < 0.0001) and % pO2 values <5 mm Hg (r = 0.63, P < 0.0001). Significant correlations were also found between mean 19FRI and the mean values for % pO2 values <10 mm Hg (r = 0.52, P = 0.001) and median pO2 (r = −0.60, P = 0.0002).

DISCUSSION

SR-4554 is a new 2-nitroimidazole bioreductive agent that was rationally designed for use as a noninvasive hypoxia marker detected by 19F MRS and potentially by positron emission tomography. Previous preclinical studies (1, 29) have established initial proof of principle for use of SR-4554 for detecting hypoxia in relatively hypoxic tumors in vivo. However, because SR-4554 was anticipated to enter clinical trials, we felt it was important to carry out a more extensive validation of the use of SR-4554 and also to use a more clinically relevant tumor model than those used previously (1, 29). We now present detailed studies of SR-4554 in the P22 tumor, which has a pO2 profile consistent with the more oxic and heterogeneous pO2 profiles encountered in human tumors (7, 8, 31). The present studies extend the previous work by evaluating the pharmacokinetic profile of SR-4554 by both HPLC-UV and 19F MRS over an entire time course, rather than at only two discrete time points. SR-4554 retention has been successfully demonstrated in the P22 tumor and has been quantified as the 3-h 19FRI. This potential parameter of hypoxia has been shown to be robust over a wide dynamic pO2 range provided by the use of agents that modulate tumor oxygenation, either up or down. The 3-h 19FRI has been shown to reflect tumor oxygenation by demonstration of correlation with pO2 measurements by the established Eppendorf polarographic electrode. In contrast to the previous studies, anesthetics have been omitted to avoid their likely influence on blood flow (58) and possible consequential effects on tumor oxygenation (59) and SR-4554 pharmacokinetics.

Our previous studies have established the pO2 profile of the P22 tumor and has been quantified as the 3-h 19FRI. This potential parameter of hypoxia has been shown to be robust over a wide dynamic pO2 range provided by the use of agents that modulate tumor oxygenation, either up or down. The 3-h 19FRI has been shown to reflect tumor oxygenation by demonstration of correlation with pO2 measurements by the established Eppendorf polarographic electrode. In contrast to the previous studies, anesthetics have been omitted to avoid their likely influence on blood flow (58) and possible consequential effects on tumor oxygenation (59) and SR-4554 pharmacokinetics.

Our previous studies have established the pO2 profile of the P22 tumor using a polarographic electrode, demonstrating a mean ± SE of median pO2 values of 11.0 ± 1.7 mm Hg with 29 ± 4.0% of pO2 values <2.5 mm Hg (30). Using pimonidazole binding studies, we have confirmed here that the P22 tumor has a nitroreductive capacity sufficient to allow bioreductive metabolism and binding of this nitroimidazole hypoxia marker. The mean ± SE percentage of immunostained area in P22...
tumors was 1.5 ± 0.5%, which is consistent with the relativelyoxic tumor profile measured polarographically. Furthermore, the pimonidazole binding in the P22 tumor was consistent with the reported levels in human carcinoma of the cervix (mean, 1.67%; range, 0.01–8.89%; Ref. 60). Use of hydralazine to induce tumor hypoxia resulted in a highly significant increase in pimonidazole binding to 37.3 ± 5.2%, confirming that increased bioreductive metabolism and binding occurs in this tumor under hypoxic conditions.

The pharmacokinetic studies of parent SR-4554 in P22 tumor-bearing SCID mice, described here, have established that parent SR-4554, measured by HPLC-UV, was eliminated rapidly from both tumor and plasma, in agreement with previous studies in EMT6 tumor-bearing female BALB/c mice (27). Thus, by 3 h only 10% (5.8 ± g/g) of parent SR 4554 remained in tumor. The complete time course of 19F signal acquired from tumor over 4 h after SR-4554 administration provided a pharmacokinetic profile of total SR-4554 in tumor detected by 19F MRS (representing the sum of parent SR-4554 and bioreduction products). Total SR-4554 exhibited a slower elimination half-life (87 min) than that for elimination of parent SR-4554 from tumor (39 min). By 3 h, the concentration of total SR-4554 (31.4 μg/ml) exceeded that of parent SR-4554 by a substantial margin (25.6 μg/ml). This indicates that 19F signal acquired from tumor at 3 h and later must have only a small (<18%) contribution from parent SR-4554 and gives an estimate of the concentration of bioreduction products retained within tumor, reflecting cellular hypoxia.

Retention of total SR-4554 in tumor was quantified as the 19FRI, defined as the ratio of concentration of total SR-4554 in tumor at a later time point (2, 3, or 4 h) to that at its peak (0.75–1 h). The advantage of using a ratio is that the parameter is independent of absolute concentrations of total SR-4554 in tumor, which could be influenced by factors other than hypoxia, including drug absorption, tumor vascularization and perfusion, and tumor size. There was considerable variation in concentrations of total SR-4554 detected by 19F MRS between different tumors at the same time points, which emphasizes the importance of using a ratio rather than a single absolute measurement. In this way, variations in pharmacokinetic and pharmacodynamic factors can be taken into account and minimized. The 3-h 19FRI was judged to be the optimal parameter of retained SR-4554 bioreduction products. This showed quite marked tumor to tumor heterogeneity in unmodulated animals (range, 0.23–0.87; coefficient of variation, 54%). The results are consistent with the known heterogeneity in oxygenation of the P22 tumor (30).

Nitroimidazoles undergo bioreductive metabolism of the nitro group, catalyzed by intracellular nitroreductase enzymes, via a number of intermediate bioreduction products before the final formation of a stable 6-electron reduced amine product (61–63). The 4-electron reduction product, the hydroxylamine, is unstable and highly reactive (64) and undergoes other reactions in addition to reduction to the amine. It can undergo highly efficient reaction with thiols (65) and thus binds to nucleophilic cellular macromolecules such as proteins, nucleic acids, and nonprotein sulfhydryl compounds (66, 67). Because of the high reactivity of the hydroxylamine, macromolecule binding occurs almost exclusively in the intracellular compartment, with very little diffusion away from cells in which it was formed (67). It is believed that it is the detection of this bound hydroxylamine that reflects cellular hypoxia. A detoxification pathway exists in which the hydroxylamine is conjugated to glutathione (68, 69). It may also undergo hydrolytic ring fragmentation yielding glyoxal (65, 70, 71), a guanidino derivative (18), or amines and aldehydes (70). Thus, the bioreduction products of nitroimidazoles have a number of fates, such that only a proportion of the administered agent will be detectable bound to poorly diffusible...

Fig. 7 Correlation analysis of mean 3-h 19FRI with means of % pO2 values <2.5 mm Hg (A), % pO2 values <5 mm Hg (B), % pO2 values <10 mm Hg (C), and median pO2 (D), for groups of P22 tumor-bearing female SCID mice that were unmodulated or modulated with hydralazine 5 mg/kg (HDZ), 100 mg/kg of CA-4-P, and 1 l/min of carbogen and 500 mg/kg of nicotinamide (C/N). n = 8–17 per group. Bars, SE.
macromolecules. Some may be bound to readily diffusible amino acids and peptides, whereas others may undergo conjugation to glutathione, or ring fragmentation to a range of potentially diffusible smaller molecules. It follows, therefore, that in the case of MRS detection of SR-4554, $^{19}$F signal acquired from tumor, representing total SR-4554, could be contributed to by any or all of the above-mentioned products of bioreduction. It should be stressed that although the precise make-up of the retained products of SR-4554 bioreduction remains unclear, the key issue is that retention should correlate with tumor hypoxia.

To validate the 3-h $^{19}$FRI as a robust parameter of tumor hypoxia, agents that modulate tumor oxygenation were used to manipulate the $^{19}$FRI in a predictable and measurable manner over a wide dynamic pO$_2$ range. Hydralazine and CA-4-P were used to increase tumor hypoxia, which was predicted to increase the $^{19}$FRI. Conversely, combined carbogen and nicotinamide were used to decrease tumor hypoxia, which was expected to decrease the $^{19}$FRI. The results overall showed that the 3-h $^{19}$FRI did change with the modulating agents in the predicted direction. However, there are a number of important practical considerations with the individual modulating agents that require discussion.

Hydralazine is an antihypertensive agent used to induce tumor hypoxia via reduced tumor perfusion (40, 48). As expected, hydralazine produced a significant increase in the 3-h $^{19}$FRI. In addition, concentrations of total SR-4554 in tumors of hydralazine-treated animals were higher at all time points compared with unmodulated animals (Fig. 4A). This is consistent with enhanced early formation and retention within tumor of SR-4554 bioreduction products, as a result of the increased tumor hypoxia. However, given that hydralazine decreases glomerular filtration rate and potentially delays renal excretion of parent SR-4554, it is also possible that the higher concentrations of total SR-4554 could be contributed to by parent SR-4554 recirculating through tumor. Furthermore, hydralazine-induced reduction in tumor blood flow could result in trapping of parent SR-4554 in tumors. Pharmacokinetic studies performed on hydralazine-treated animals showed that hydralazine does delay clearance of parent SR-4554 from both plasma and tumor. Nevertheless, concentrations of total SR-4554 in tumor consistently exceeded those of parent SR-4554, and the relative contribution of parent SR-4554 decreased with time such that at 3 h, parent SR-4554 contributed to total SR-4554 by only 34%. The 3-h $^{19}$FRI parameter takes into account variations in parent SR-4554 distribution into tumors by normalizing $^{19}$F signal at 3 h against the peak signal at 45–60 min. However, when considering modulation with hydralazine, because it alters the pharmacokinetic profile of parent SR-4554 it is necessary to account for overall tumor exposure to parent SR-4554. Assessment of this by the ratio of bioreduced SR-4554 to the area under the parent SR-4554 tumor concentration-time curve demonstrates a higher value for hydralazine-treated compared with unmodulated tumors. Thus, although hydralazine undoubtedly has some effect on the pharmacokinetic profile of parent SR-4554, the increase in 3-h $^{19}$FRI appears to be in larger part because of hypoxia-induced formation and retention of SR-4554 bioreduction products within tumor.

Similar to hydralazine, CA-4-P administration resulted in an increase in 3-h $^{19}$FRI. High concentrations of total SR-4554 in tumor were observed, indicating that parent SR-4554 had successfully reached tumor before the onset of CA-4-P-induced effects on tumor vessels. CA-4-P has little effect on renal blood flow (53), and therefore, it is unlikely that nonexcreted parent SR-4554 was recirculating through tumor and contributing to the high total SR-4554 concentrations. It is possible that some contribution could be made by parent SR-4554 trapped in tumor after CA-4-P-induced decreases in tumor blood flow. However, it equally feasible that this trapped parent SR-4554 may have undergone hypoxia-induced metabolism to bioreduction products. Thus, the increase in 3-h $^{19}$FRI is entirely consistent with a hypoxia-induced enhancement of early formation and retention of SR-4554 bioreduction products. Moreover, the CA-4-P-induced increase in retention of total SR-4554 in tumor offers a novel way of monitoring the antivascular effects of CA-4-P when used therapeutically (16).

Carbogen and nicotinamide used together reduce tumor hypoxia (46) by increasing tumor blood flow (72). Concentrations of total SR-4554 in tumors of modulated animals were slightly higher at all time points than those in unmodulated animals, which could be attributable to increased tumor blood flow increasing delivery of parent SR-4554 to tumor. However, the shorter elimination half-life of total SR-4554 from tumors of modulated compared with unmodulated animals is consistent with decreased formation and retention of SR-4554 bioreduction products as a result of the reduction in tumor hypoxia. This is reflected by the observed lower 3-h $^{19}$FRI.

As a further validation of the $^{19}$FRI as a parameter of tumor hypoxia, comparison was made with an established method of oxygen measurement, the Eppendorf polarographic electrode. Correlation analysis was performed between mean values for the 3-h $^{19}$FRIs and polarographically measured pO$_2$ parameters over the wide pO$_2$ range provided by groups of modulated as well as unmodulated animals (Fig. 7). Highly significant correlations were obtained for all analyses. The most significant relationships were those between $^{19}$FRI and % pO$_2$ values <2.5 mm Hg ($P < 0.0001$) and <5 mm Hg ($P < 0.0001$), corresponding with the range of radiobiological hypoxia. In addition, a statistically significant correlation was observed between the 3-h $^{19}$FRI and % pO$_2$ values <2.5 mm Hg for individual tumors ($r = 0.43, P < 0.01; n = 36$). Our findings are consistent with those of a previous study that demonstrated a significant correlation between pimonidazole binding and % pO$_2$ values <10 mm Hg across groups of animals whose tumor oxygenation had been modulated by various maneuvers similar to those used in the present study (38). However, if the $^{19}$FRI is to be used in a clinical setting to make decisions regarding individual patient treatment, it would be desirable to show a correlation with polarographically measured pO$_2$ in individual unmodulated tumors. In the present study, no such correlation was demonstrated (data not shown), and indeed a similar study of the relationship between EF5 binding and polarographic pO$_2$ in individual unmodulated tumors also failed to show a significant correlation (73). This lack of correlation is probably because of the major differences in the ways in which the two methods measure oxygenation. The polarographic electrode measures extracellular pO$_2$, assessing the environment of several hundred cells per single pO$_2$ reading (30). In contrast, nitroimidazoles measure intracellular pO$_2$ on an individual cell basis. Thus, it
may be more appropriate to compare nitroimidazole binding with another technique that assesses hypoxia at a single-cell level such as radiobiological hypoxic fraction. Two studies have demonstrated a correlation between nitroimidazole binding and radiobiological hypoxic fraction in individual unmodulated tumors (73, 74). It should be noted, however, that the true test of any new parameter of tumor hypoxia is whether it can predict a poor clinical outcome after anticancer treatment in patients whose tumors have been identified by that parameter to be hypoxic. This can only be determined by a prospective clinical study.

An important aspect of the evaluation of SR-4554 as a hypoxia marker in humans will be the sensitivity of MRS detection of SR-4554 in tumors. It will be necessary to achieve concentrations of SR-4554 in human tumors that are sufficient to be detectable by MRS. This should be possible by administration of adequate doses of SR-4554. However, nitroimidazoles used as radiosensitisers in humans have been associated previously with side effects including nausea, vomiting, and peripheral neuropathy (19). The key issue, therefore, is whether nontoxic doses of SR-4554 will be sufficient to reliably detect tumor hypoxia in humans by MRS. A Phase I study of SR-4554 is required to demonstrate toxicity and establish a maximum tolerated dose, because predictions regarding drug safety cannot be made from animal studies. This has now begun (see below), and initial results indicate that retention of SR-4554 can be demonstrated in human solid tumors (75).

In summary, we have provided evidence that SR-4554 is bioreduced and retained within the P22 carcinosarcoma, a tumor with an oxygenation profile similar to that encountered in human tumors, and that this retention can be quantified using the 3-h $^{19}$FRI. This potential parameter of tumor hypoxia has been shown to be robust over a wide $pO_2$ range and has been further validated by correlation with $pO_2$ measured by polarographic electrode. The possibility that the modulatory agents could have produced changes in the 3-h $^{19}$FRI by mechanisms other than those involving altered tumor oxygenation has been discussed. However, we have provided clear evidence that use of hydralazine, while demonstrably affecting the pharmacokinetic profile of SR-4554, nevertheless results in increased formation and retention of SR-4554 bioreduction products. Moreover, it seems unlikely that modulatory agents acting by such distinct mechanisms would consistently increase and decrease the 3-h $^{19}$FRI with polarographically measured tumor $pO_2$ in the predictable way observed, other than via $pO_2$ modulation.

The positive nature of these results is supportive of the further development of SR-4554 as a noninvasive probe for determining the hypoxic status of human tumors, and SR-4554 has now entered Phase I study at the Royal Marsden Hospital in association with the Institute of Cancer Research, with encouraging early results.

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