

## Novel Non-invasive Probes for Measuring Tumor-hypoxia by $^{19}\text{F}$ -Magnetic Resonance Spectroscopy ( $^{19}\text{F}$ -MRS). Studies in the SCCVII/C3H Murine Model

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**Abstract.** *Background:*  $^{19}\text{F}$ -labeled 2-nitroimidazoles bound to hypoxic cells in tumors are known to be useful probes for measuring hypoxia since they can allow for their non-invasive detection by  $^{19}\text{F}$  nuclear magnetic resonance, provided that they do not lose  $^{19}\text{F}$  during their hypoxia-mediated metabolism. Two such compounds, *N*-(*m*-trifluoromethylbenzyl)-3-(2-nitro-1-imidazolyl)-propylamine hydrochloride (*mTFN-1*) and 5,6-dimethyl-4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-trifluoromethylpyrimidine hydrochloride (*CF3PM*) were selected from a series of analogs, for their *in vivo* evaluation, based on their high solubility in saline and low toxicity in mice. *Materials and Methods:* MRS experiments were performed in anesthetized C3H mice bearing SCCVII tumors in their flanks. Fluorinated compounds, *mTFN-1* or *CF3PM*, were injected intraperitoneally (*i.p.*) at a dose of 110 or 150 mg/kg, respectively, in 0.75 mL saline. A 0.9 cm surface coil tuned to fluorine frequency was positioned directly over the tumor, the head, or the liver and 1800 transients were collected over 20 min in a Bruker Omega 4.7 T instrument. Spectroscopic measurements were taken at 2, 7 and 19 h post injection of the fluorinated drug. *Results:* *CF3PM* was detected in the plasma up to 2 h post injection with maximum concentration observed 30 min post administration. In the MRS studies, *mTFN-1* signal in the tumor was 68.8, 86.8 and 27.2% of the reference at 1-2, 6-7 and 18-19 h post injection, respectively. The corresponding values in the brain were 0, 125.7 and 26.6%, respectively, whereas the corresponding values in the liver were 359.3, 307.7 and 0%, respectively. *CF3PM* signal in the tumor was 3.3, 57.7 and 7.1% of the reference at 1-2, 6-7 and 18-19 h post injection, respectively. The corresponding values in the liver were 267.6, 60.5 and 0%, respectively. No *CF3PM* signal

was detected in the brain at any time interval. *Conclusion:* These results suggest that *CF3PM* could be used as a potential probe for measuring hypoxia in tumors by  $^{19}\text{F}$ -MRS.

Evidence from clinical investigations strongly suggests that tumor hypoxia is a serious detriment to curative anticancer therapies in at least some human cancer types. The identification and quantification of hypoxic cells in the tumors of patients undergoing treatment therefore is of considerable importance (1). Bioreductively activated nitroimidazoles have been used as probes for measuring tumor-hypoxia by immunohistochemistry (2). However, non-invasive techniques for measuring hypoxia in tumors, such as with magnetic resonance spectroscopy (MRS), are highly desirable.

$^{19}\text{F}$ -MRS probe molecules are particularly useful in biology because of the relatively high sensitivity for detection (0.83% that of  $^1\text{H}$ ) and low endogenous background. Therefore, appropriately fluorinated 2-nitroimidazoles have been synthesized for the non-invasive detection of hypoxia in human cancers (3-5).

Nitroimidazoles undergo a hypoxia-dependent, one electron reduction catalyzed by cellular reductases, resulting in reactive intermediates that form covalent adducts with cellular components (6, 7). Since the adducts are cleared at a slower rate compared to the parent 2-nitroimidazole, the degree of hypoxia within the tumors can be assessed by measurement of residual (metabolized) drug after "washout" of the original compound. However, only those probes with multiple fluorine substitution or which can be given in relatively high doses (in the order of 0.1 mmol of magnetically equivalent fluorine atoms per kilogram) are suitable for MRS/magnetic resonance imaging studies (8). Other important design criteria for these probes include their chemical/biological stability (other than due to hypoxic bioreduction), pharmacokinetic/toxicity considerations such as tumor-to-plasma and brain-to-plasma partition coefficients (which are in turn related to lipophilicity/ hydrophilicity) as well as normoxic toxicity. In addition to these considerations, signal attenuation due to macromolecular binding and its influence on the

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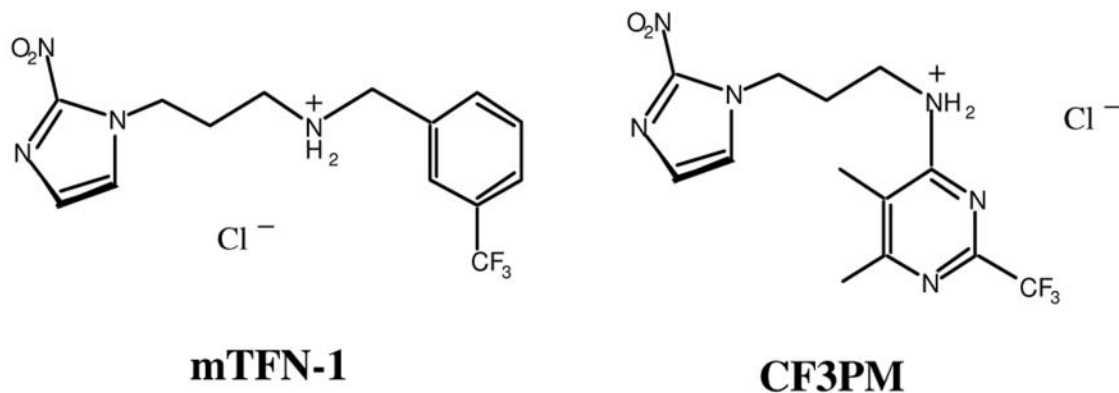


Figure 1. Chemical structure of mTFN-1 and CF3PM.

stoichiometry of the retention of these compounds is relevant to the quantitation of hypoxia in tumors (8).

Taking into account the above considerations, we have synthesized a small series of fluorinated nitroimidazole-based compounds, carrying three or six magnetically equivalent fluorines, as potentially useful hypoxia-markers by  $^{19}\text{F}$ -MRS. Two such compounds, *N*-(*m*-trifluoromethylbenzyl)-3-(2-nitro-1-imidazolyl)-propylamine hydrochloride (mTFN-1) and 5,6-dimethyl-4-[3-(2-nitro-1-imidazolyl)-propylamino]-2-trifluoromethylpyrimidine hydrochloride (CF3PM) were selected for their *in vivo* evaluation, based on their high solubility in saline and low toxicity in mice (Figure 1).

## Materials and Methods

**Chemicals.** mTFN-1 and CF3PM were synthesized in house (see accompanying paper) and identified by UV-Vis,  $^1\text{H}$  and  $^{19}\text{F}$ -NMR spectroscopy and HRMS. For *in vivo* testing, mTFN-1 or CF3PM, were injected intraperitoneally (*i.p.*) at a dose of 110 or 150 mg/kg, respectively, in 0.75 mL saline.

**Tumors.** SCCVII squamous carcinoma tumor-cells ( $5 \times 10^4$  cells in 0.05 ml; a gift from Dr. D. Siemann, University of Florida, Gainesville, FL) were inoculated subcutaneously in the leg of 18-20 g male C3H mice (Jackson Laboratories), which were housed under germ-free conditions. All studies were conducted according to the guidelines set by the Evanston Northwestern Healthcare Institutional Animal Care. NMR studies were initiated in anesthetized mice when the tumor mean diameter was 8-10 mm.

**$^{19}\text{F}$ -NMR studies.** A 0.9 cm surface coil tuned to fluorine frequency was positioned directly over the tumor, the head, or the liver (the latter after sacrifice). A reference solution of 5 mM NaF in water in a 1 cm spherical bulb was positioned on the surface coil and the animal mounted on a Plexiglass cradle. Body temperature was maintained with water circulating through a heating pad at  $37^\circ\text{C}$ . Animals were allowed to maintain spontaneous respiration. The cradle was positioned inside a Bruker Omega 4.7 T (Fremont, CA, USA) magnet with the tumor at the isocenter. Spectroscopic

measurements were carried out at 2, 7 and 19 h post injection of the fluorinated drug. For each experiment, 1800 transients were collected over 20 min. The animals were euthanized at the end of the experiment. Excised tumor, brain, and liver tissues were examined at the same time intervals. The resonances of fluorinated metabolites and the NaF reference were integrated for calculation of absolute metabolite concentrations.

**Pharmacokinetic studies.** Preliminary pharmacokinetic studies were also performed with CF3PM at 150 mg/kg (*i.p.*), in BALB/c female mice. Blood samples ( $2 \times 10 \mu\text{l}$ ) were withdrawn from the tail vein of the mouse without anesthesia, at various time intervals and were centrifuged immediately to obtain plasma. Then, MeOH ( $90 \mu\text{l}$  per  $10 \mu\text{l}$  of plasma) was added and the samples were kept at  $-80^\circ\text{C}$  until HPLC analysis. A reverse phase Econocil C18 5U column, 250 mm long (Altech) was used; 70% MeOH in 10 mM  $\text{Na}_2\text{HPO}_4$ , 5 mM dibutyl-ammonium phosphate, 7.5 mM heptane sulphonic acid at pH 3.5 was the mobile phase. The detector was set at 330 nm. NLQZ-1 was used as an internal standard. Sensitivity was set at 0.002. Flow rate was 1 ml/min.

## Results

Both compounds did not cause weight loss or lethality in BALB/c mice up to 150 mg/kg, the highest tested dose. However, mTFN-1 at 120 and 150 mg/kg, given *i.p.*, demonstrated some signs of neurotoxicity 20 min post injection (see accompanying paper). Therefore, mTFN-1 and CF3PM were given *i.p.* at 110 and 150 mg/kg, respectively, in the described NMR studies.

CF3PM was detected in the plasma up to 2 h post injection with the peak plasma concentration observed 30 min post injection. Recovery from plasma was poor, but an optimized methodology was not attempted. An HPLC chromatogram is shown in Figure 2.

The data from the NMR (MRS) studies are summarized in Table I, whereas an example is given for CF3PM spectra in Figure 3.

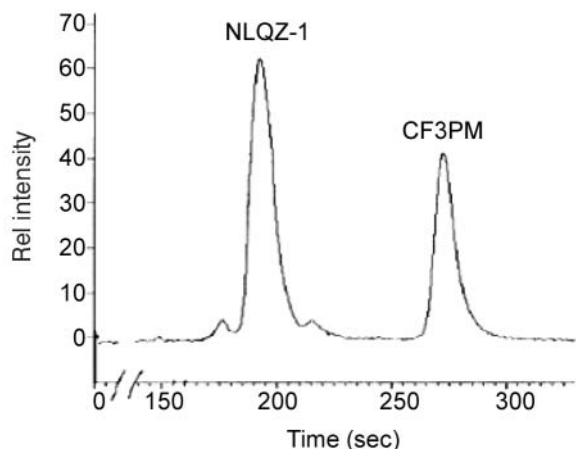


Figure 2. HPLC detection of CF3PM in the plasma of BALB/c mice. NLQZ-1, a 2-nitroimidazole analog was used as an internal standard. For further details see Materials and Methods.

The mTFN-1 signal in the tumor was 68.8, 86.8 and 27.2% of the reference at 1-2, 6-7 and 18-19 h post injection, respectively. The corresponding values in the brain were 0, 125.7 and 26.6%, respectively, whereas the corresponding values in the liver were 359.3, 307.7 and 0%, respectively. Therefore, mTFN-1 was rapidly accumulated in tumors, but it was also accumulated in the brain, at a slower rate.

CF3PM signal in the tumor was 3.3, 57.7 and 7.1% of the reference at 1-2, 6-7 and 18-19 h post injection, respectively. The corresponding values in the liver were 267.6, 60.5 and 0%, respectively. No CF3PM signal was detected in the brain at any time interval. These results suggest that CF3PM could be detected in the tumor up to 19 h post injection. In addition, no parallel accumulation was observed in the brain, suggesting a good safety profile for this compound and its potential use as a hypoxia marker with <sup>19</sup>F-MRS technology.

## Discussion

The use of nitroimidazoles as hypoxia markers requires only a single administration of the agent. Cumulative doses are, therefore, considerably lower than those required for radiosensitization over a course of radiotherapy, reducing the risks of side effects encountered with multiple dose schedules. Currently, three nitroimidazole-based bioreductive agents are undergoing clinical evaluation as hypoxia markers: pimonidazole (9, 10) and EF5 (11, 12) by immunohistochemistry in biopsy specimens, and SR-4554 by non-invasive <sup>19</sup>F-MRS detection (3).

For a non-invasive probe of tumor hypoxia by <sup>19</sup>F-MRS detection, two factors are important: a) minimal or manageable toxicity and b) sensitivity, otherwise maximum

Table I. <sup>19</sup>F signal levels (% of the reference's integral) in tissues of C3H mice following an *i.p.* administration of mTFN-1 (110 mg/kg) or CF3PM (150 mg/kg).

Tissue	mTFN-1			CF3PM		
	1-2 h	6-7 h	18-19 h	1-2 h	6-7 h	18-19 h
Tumor	68.8	86.8	27.2	3.28	57.7	7.1
Brain	ND	125.7	26.6	ND	ND	ND
Liver	359.3	307.3	ND	267.6	60.5	ND

A 0.9 cm diameter surface coil tuned to <sup>19</sup>F frequency was used to detect signals at the indicated post injection time intervals, in SCCVII tumors, brain and liver of C3H mice. A 5 mM NaF aqueous solution (placed in a plastic bulb located directly above the surface coil) was used for quantitation of the signal. Trifluoroacetic acid sodium salt (TFA) was used as the fluorine chemical shift reference for all experiments. ND: not detectable.

signal to noise ratio. Experience with the cytotoxic agent 5-fluorouracil (5FU, which has one <sup>19</sup>F) indicates that it can be detected by <sup>19</sup>F MRS in tumors in humans after *i.v.* doses of 300-1000 mg/m<sup>2</sup>, which is equivalent to 0.0615-0.205 mmol/kg for an average-size adult (3, 13). Our data indicate that both CF3PM and mTFN-1 fulfill the sensitivity and minimal toxicity criteria. Thus, CF3PM, given at 150 mg/kg, was not toxic and in terms of magnetically equivalent fluorine atoms per kilogram, this dose was 1.18 mmol, namely 19.2 fold greater than the lowest detectable dose of 5FU. Moreover, this is simply the highest tested dose and it does not represent its MTD. Similarly, mTFN-1 at 110 mg/kg demonstrated manageable toxicity (some sedative effects only) and this dose represents 0.9 mmol of magnetically equivalent fluorine atoms per kilogram, 14.6 fold greater than the lowest detectable dose of 5FU.

From our previous *in vitro* studies in V79 cells, we know that both compounds behave as hypoxia-selective cytotoxins with a hypoxic selectivity of 5.3 and 15.5 for mTFN-1 and CF3PM, respectively. Therefore, the signal detected at 6-7 h post injection for each compound, most likely represents bioreduced metabolites attached to cellular macromolecules rather than parent compounds. On the contrary, signals at 1-2 h post injection are most likely due to the parent compounds, as has been demonstrated before with the fluorinated hypoxia marker SR-4554 (14). The ratio of <sup>19</sup>F signal levels determined by MRS at 6-7 h relative to 1-2 h (<sup>19</sup>FRI), should be indicative of the degree of hypoxia in a specific tumor (14). This ratio was higher (17.6) in the case of CF3PM, compared to 1.3 for mTFN-1, consistent with the better hypoxic selectivity observed for CF3PM *vs.* mTFN-1.

CF3PM does not accumulate in the brain at any observed time post administration and therefore offers an advantage over SR-4554, which was detected in the brain at 1.08 h post administration (14). CF3PM's lack of ability to cross the

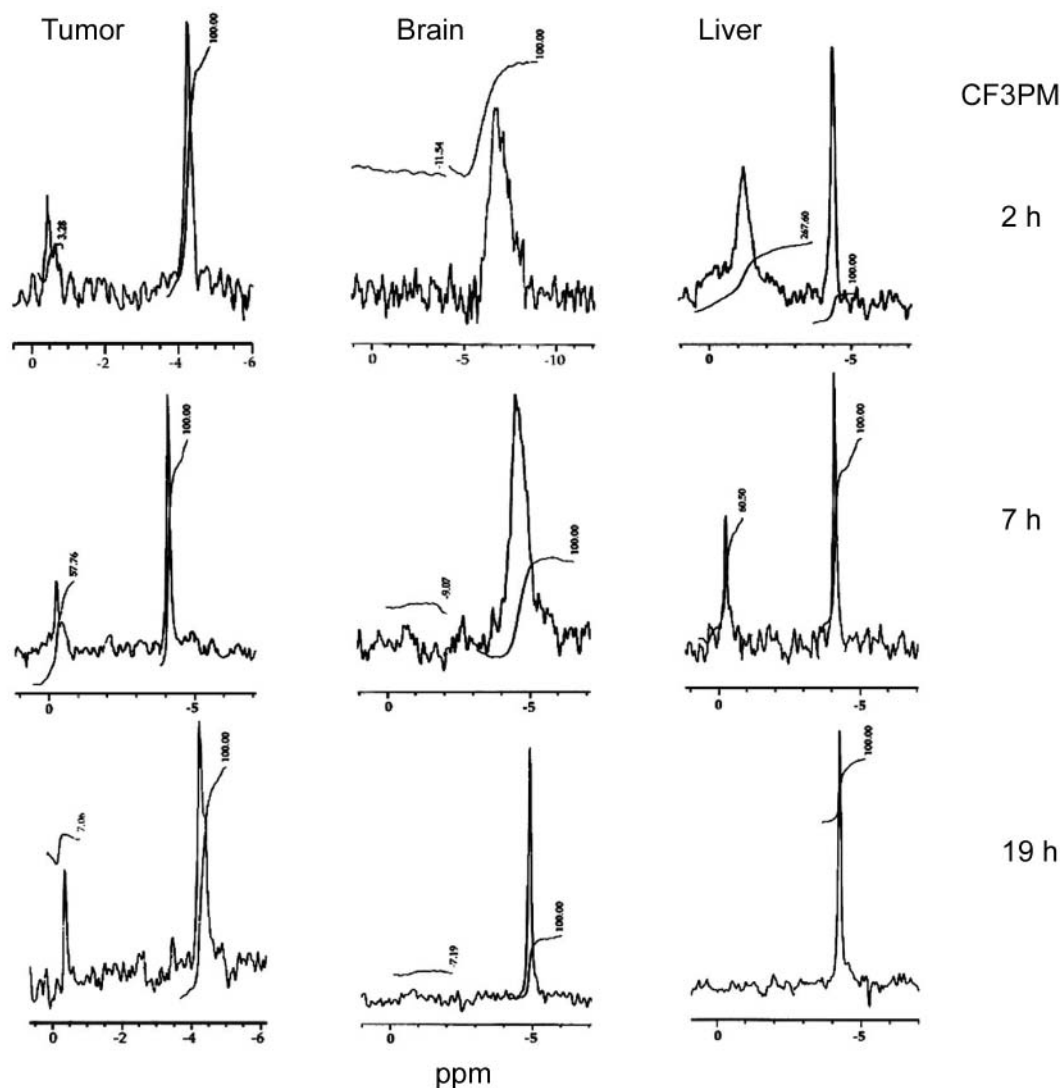


Figure 3. NMR-spectra taken from tumor, brain and liver of SCCVII tumor-bearing C3H mice at various time-intervals post *i.p.* injection of CF3PM (150 mg/kg). NaF was used as reference and its integral was assigned as 100.

blood-brain barrier presumably explains its lack of sedative effects or neurotoxicity. In addition, CF3PM, as a hydrochloride salt of a pyrimidinic system, provides better solubility than the amidic SR-4554.

Future studies are necessary to demonstrate how measurements of hypoxia in tumors by other methods compares with measurements from the  $^{19}\text{F}$ -MRS methodology by using the CF3PM probe.

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